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Receptors for the detection of L-amino acids and IMP by mouse taste sensory cells

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RECEPTORS FOR THE DETECTION OF L-AMINO ACIDS AND IMP BY MOUSE TASTE SENSORY CELLS

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ABSTRACT

The sense of taste is one of the most important factors in regulating ingestive decisions. This is central to a number of disease conditions including but not limited to obesity, diabetes, anorexia, hypertension, coronary artery diseases and malnutrition. The detection of the molecules eliciting taste qualities in food is mediated by the coordinated actions of distinct types of taste sensory cells (TSCs) housed in taste buds within specialized papillae throughout the oral cavity. Taste receptors in the taste sensory cells that detect food molecules are the key players in selecting dietary nutrients. One such example is L-amino acids, a critical part of one's diet.

L-glutamate is the prototypical umami compound and is known to increase palatability of food. A unique characteristic of umami taste is the response potentiation of glutamate by 5' ribonucleotide monophosphates, such as inosine 5' monophosphate (IMP), which is also capable of eliciting an umami taste. Candidate receptors for umami taste include a heterodimer T1r1+T1r3, brain variants of mGluR1 and mGluR4, and the truncated variants of mGluR1 and mGluR4. Studies using heterogeneous expression of T1r1+T1r3 suggest it is an umami and a broadly tuned L-amino acid receptor. While much attention is devoted to understanding glutamate transduction, the detection mechanisms for other L-amino acids by TSCs are less well understood. Here calcium imaging of isolated TSCs and taste cell clusters from the circumvallate and foliate papillae of C57BL/6J and T1r3 knockout mice was performed to determine if other receptors are involved in the detection of L-amino acids and IMP. Ratiometric imaging with Fura-2 was used to study calcium responses to IMP and four L-amino acids (monopotassium L-glutamate, L-serine, L-arginine, and L-glutamine) with and without IMP. The results of these experiments showed that the response patterns elicited by L-amino acids varied significantly across TSCs. Only a small subset of cells responded to all stimuli. Interestingly, L-amino acids other than glutamate elicited synergistic responses in a subset of TSCs. Additionally IMP alone elicited a response in a large number of TSCs. Our data indicate that synergistic and non-synergistic responses to L-amino acids and IMP are mediated by multiple receptors or possibly a receptor complex.

Next the roles of mGluR1 and mGluR4 in the detection of the IMP and L-amino acids were investigated. Selective agonists for mGluR1, (*RS*)-3, 5-dihydroxyphenylglycine (DHPG; a group I mGluR agonist), and mGluR4, L-(+)-2-amino-4-phosphonobutyric acid (L-AP4; a group III mGluR4 agonist) elicited responses in TSCs. In addition, TSCs responsive to these agonists were also responsive to L-amino acids and IMP. More importantly, selective antagonists against different mGluRs such as (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA; a group I mGluR antagonist), and (*RS*)-α-methylserine-*O*-phosphate (MSOP; a group III mGluR antagonist) significantly suppressed L-amino acid- and IMP-mediated responses in TSCs of T1r3 knockout mice. Collectively, these data provide evidence for the involvement of taste and the brain variants of mGluR1 and mGluR4 in L-amino acid and IMP taste responses in mice, and support the hypothesis that multiple receptors contribute to the IMP and L-amino acid tastes.

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DEDICATION

This is dedicated to

my mother Ushasi Pal Choudhuri and my father Subrata Pal Choudhuri

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

Chapter 1 : TASTE TRANSDUCTION – A COMPREHENSIVE LITERATURE REVIEW

1.1 Taste

1.1.1 Sense of Taste:

The sense of taste, also called gustation, is important for guiding an organism to identify nutritious edible compounds while avoiding toxic ones. In other words, the sense of taste tells an organism what to or not to eat. For humans, what we consume depends on many different factors, such as the color and appearance of the food, the aroma, temperature, taste, flavor, and even the physiological and mental condition of an individual. Among all these, one major deciding factor is the taste of the food. From a human perspective, the sense of taste can be qutatively or physiologically described as perceiving one of the five "basic taste": sweet, salt, sour, bitter, and finally umami, the latest addition to the basic taste list. The idea of basic taste came from the "taste tetrahedron" model of a German psychophysist Hans Hunnings (McC. Gamble, 1922). A "basic taste" was attributed to have three main qualities: 1) the taste must be unique by itself and cannot be created by a combination of other taste qualities, 2) must have a unique perception that can be identified by a single receptor on the taste epithelium, and 3) the ability to detect the substance must be innate. However, today the definition of a basic taste is quite ambiguous and does not completely hold the same value for defining a taste, as many of the basic tastes can be identified by multiple receptors (Chandrashekar et al., 2000; Damak et al., 2003; Maruyama, Pereira, Margolskee, Chaudhari, & Roper, 2006; Yasuo, Kusuhara, Yasumatsu, & Ninomiya, 2008; Yasumatsu et al., 2012)

Each of the basic tastes is believed to represent different nutritional and physiological requirements or pose potential dietary hazards (Figure1.1).

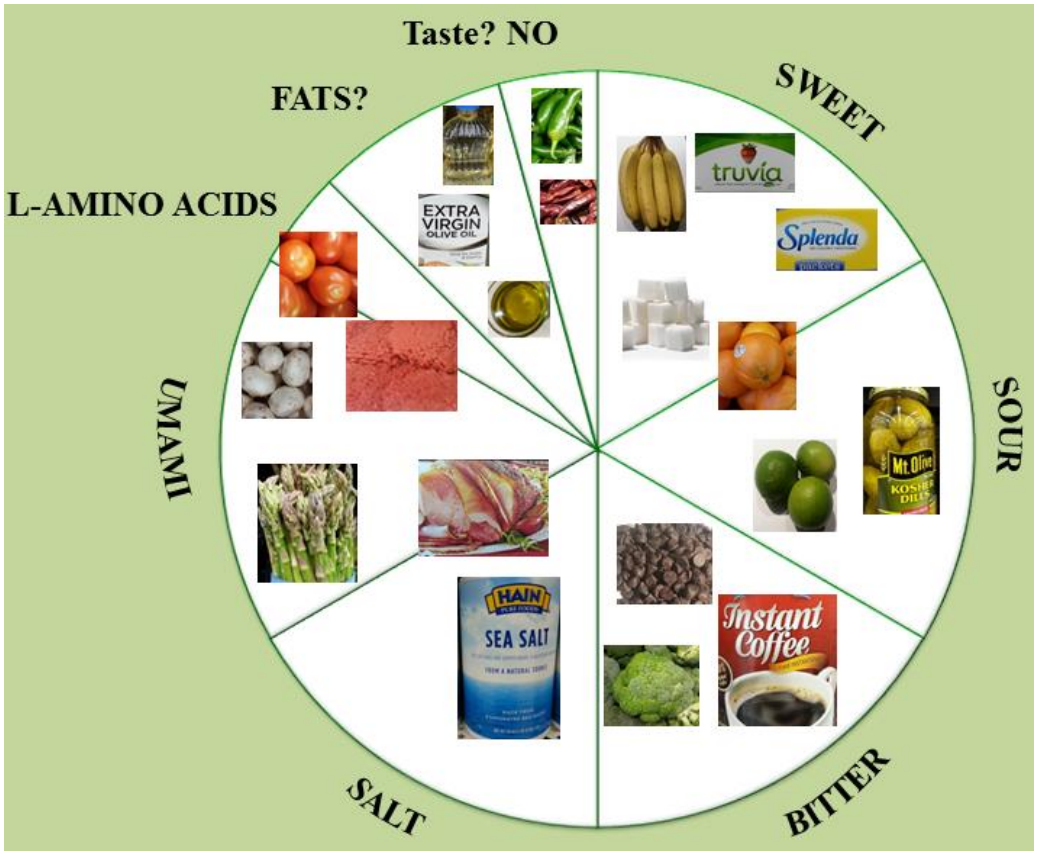


Figure 1.1: Basic taste and others.
Five basic taste qualities – Sweet, sour, salt, bitter, and umami are considered basic tastes. Sweet taste gives the signal for carbohydrate/energy in food and is generally pleasant. Sour taste signals for acids in food and is innately aversive. Bitter taste is thought to protect against ingestion of toxic compounds. Many foods also taste sour or bitter and we learn to accept and even crave them. Salt taste is generated mainly by Na^+. Umami taste is elicited by L-amino acid glutamate and 5' ribonucleotides, is found in free form in many different foods, and is considered to signal for protein. Other L-amino acids are also found in the same food as of umami and generally elicit a complex taste. Heat of chili peppers or the coolness of mint is sometimes confused with taste, but they are actually somatosensation. Fat falls in the middle of somatosensation and taste. While there are specific receptors that detect fat, some component of fatty sensation is also thought to be elicited as somatosensation. Note: Most of the foods that we consume generally elicit a complex taste that includes multiple basic tastes and somatosensory sensations.

Sweet: The sweet taste primarily gives the signal for simple carbohydrates in food and serves as a source of quick energy. The prototypical compound that elicits sweet taste is sucrose, although there are several natural and artificial compounds that can elicit a

sweet taste. Sweetness is generally regarded as pleasurable experience and is probably the taste with most ancient evolutionary beginnings. Chemical detection of sugar or sweetness manifests as chemotaxis even in prokaryotes like *E.coli.* Newborn human infants also show an inclination for food with high sugar concentrations and even prefer solutions that are sweeter than lactose, the sugar found in breast milk (Maller & Turner, 1973).

Salt: Salt taste signals sodium (Na^+) in food and helps govern the intake of sodium and other salts essential for maintaining several physiological functions. The prototypical compound to elicit a salt taste is table salt, sodium chloride (NaCl). Although Na^+ is essential for regulation of several physiological functions, its amount in the cell and in blood needs to be tightly regulated. Therefore, little to moderate salt is pleasurable, but too much of it is aversive.

Sour: Sour taste signals the presence of H^+ ions in food. It primarily indicates dietary acids. In general, sour taste is aversive to prevent over-consumption of dietary acids, and helps maintain acid-base balance in the body. Additionally, spoiled foods are also often acidic, hence sour taste helps us against potential consumption of rotten substances. The prototypical compound that elicits a sour taste is citric acid. However, many fruits also contain other organic acids.

Bitter: Bitter taste has evolved to allow the detection of toxins in the environment primarily produced by plants. Bitterness is innately aversive and it signals toxic substances in the food. Bitter compounds have the lowest detection threshold and are the most sensitive of all the taste qualities. The prototypical compound to elicit a bitter taste

is quinine. Even though bitter and excess sour tastes are generally aversive, people learn to overcome this innate response and even seek out certain bitter and sour compounds such as caffeine, dark chocolate, lemon etc.

Umami: The umami taste signals protein in food and is generally attributed to a few free L-amino acids, such as glutamate and 5' ribonucleotides, such as inosine 5' monophosphate (IMP), and guanosine 5' monophosphate (GMP) in food. The term umami, which also translates as savory or meaty taste, was coined by Japanese scientist Dr. Kikunae Ikeda in 1908 (Ikeda, 2002). The prototypical compound for umami taste is the monosodium glutamate (MSG). A unique defining characteristic of umami taste is the synergistic response potentiation of L-glutamate taste in the presence of IMP or GMP. Food containing high amounts of free L-glutamate and IMP and/or GMP, generally elicits the umami taste. Some examples of umami tasting foods are fish, meat, vegetables such as a ripe tomato, asparagus, spinach, celery, green tea, mushroom, cheese, soy sauce, etc. One interesting characteristic of umami taste is that it is known to increase palatability of food, thus it has potential for making food more or less desirable (Bellisle et al., 1991; Yamaguchi & Ninomiya, 2000).

Apart from these 5 basic tastes, there are likely additional qualities that might also take a seat in the list of basic tastes. These include fatty and metallic taste (Lawless et al., 2004; Lawless, Stevens, Chapman, & Kurtz, 2005; Mattes, 2009a, b; Cartoni et al., 2010). Additionally, carbonation has been suggested to have a unique taste and has a dedicated group of receptors in the taste buds (Chandrashekar et al., 2009).

Taste of L-amino acids: In one word, the tastes of L-amino acids are described as complex taste (Kawai, Sekine-Hayakawa, Okiyama, & Ninomiya, 2012). Like L-glutamate, other L-amino acids are found in free form in many different foods and are important nutrients. There are specific receptors in the taste buds that can detect L-amino acids. However, all L-amino acids do not explicitly elicit the same taste and therefore are not included in the umami taste category. For example, a single L-amino acid (e.g. serine) can elicit different taste qualities (sweet at low concentration and umami at high concentration) in a concentration-dependent manner (Kawai et al., 2012).

1.1.2 Taste and Physiological Functions:

In addition to guiding food intake, the sense of taste also serves other physiological functions collectively called the cephalic phase response (Chaudhari & Roper, 2010). The physiological changes start with the recognition of food. Activation of taste buds in the oral cavity gives signals to initiate physiological reflexes to prepare the gut to release digestive enzymes, initiate peristalsis, increase mesenteric flow, all of which together prepares the gut for absorption of nutrients. It also initiates metabolic adjustments in the body, such as release of insulin, activation of brown adipose tissue and increase in heart rate (Giduck, Threatte, & Kare, 1987; Mattes, 1997).

1.1.3 Taste Confusion:

Taste is most commonly confused with flavor. The sense of taste is solely mediated by taste sensory cells (TSCs) located in the oral cavity. Activation of these TSCs by molecules in the food initiates taste responses. In comparison, flavor is a combination of taste and retro-nasal olfaction, where the olfactory signal is generated by

activation of neurons located in a specialized patch of nasal epithelium by volatile compounds. One great example of this confusion is the sensation that comes from eating chocolate. The taste part mainly comes from the bitterness of coco powder and the sweetness of added sugar, but the main experience comes from the aroma of the compounds in chocolate.

Taste sensations are frequently confused with somatosensory sensations such as the spiciness or the heat of chili pepper (main compound: capsaicin) or black pepper (active compound: piperine) and the cool sensation of menthol. The hot sensation triggered by chili peppers, black pepper, and other spices like ginger is an important feature in many cuisines. These sensations are not considered as taste strictly because these sensations are not generated by activation of taste cells in the taste buds, rather are mediated by activation of ion channels (such as TRPV1 and TRPA1 receptors) located in the somatosensory nerve fibers (Caterina et al., 1997; McKemy, Neuhausser, & Julius, 2002). However, the compounds generating heat or cold sensations may also modulate taste function by stimulating interactions between somatosensory nerve fibers and taste buds (Wang, Erickson, & Simon, 1995; Whitehead, Ganchrow, Ganchrow, & Yao, 1999; Waterer, 2012) (Figure 1.1).

For long time recognition of fatty taste was mainly attributed to the function of its texture and somatosensory origin. Fatty acids are important dietary nutrients and are found in many foods. Recent evidence suggests that fatty acids may be detected by specific receptors expressed in the TSCs in the taste buds and thus could be recognized as a sixth basic taste (Gilbertson, Liu, York, & Bray, 1998; Gilbertson, Liu, Kim, Burks, &

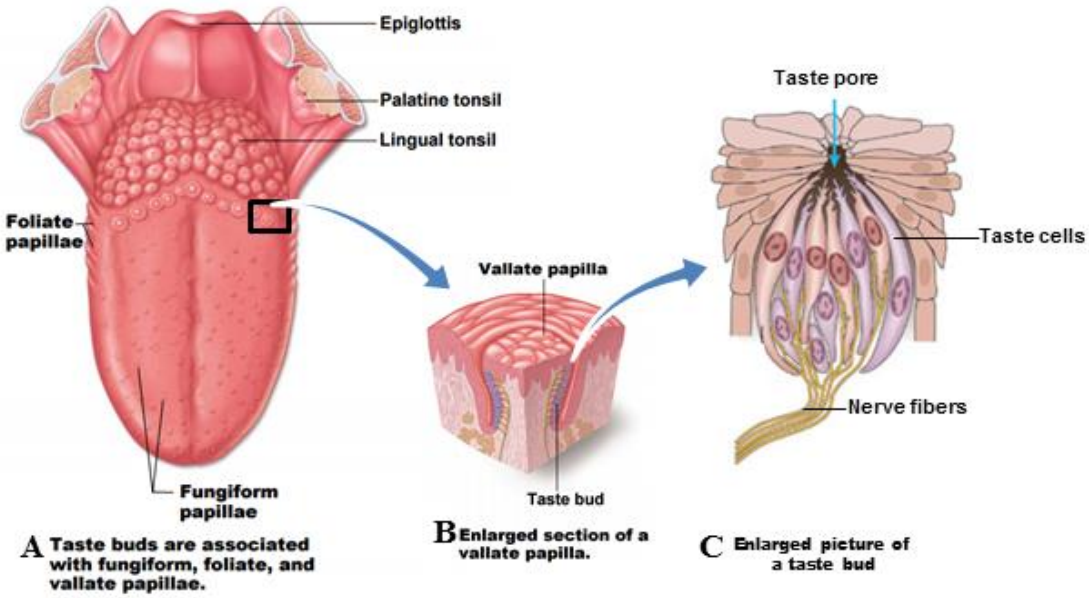
Hansen, 2005; Laugerette et al., 2005; Sclafani, Ackroff, & Abumrad, 2007; Mattes, 2009a, b) (Figure 1.1).

1.2 Taste System

The primary sensory organ involved in the sense of taste is the taste bud. The primary units within taste buds that mediates detection of taste stimuli are taste sensory cells (TSCs). These are clustered in an onion-shaped structure of the typical taste bud. These taste buds are located in three specific regions of the taste epithelium called taste papillae. Taste papillae in the anterior portion are called fungiform papillae, in the posterior region are called circumvallate, and in the lateral-posterior portion they are called foliate papillae. Taste buds are also located in the epithelium of palate, oropharynx, larynx, and upper surface of the esophagus (Kinnamon & Roper, 1987).

1.2.1 The Structure of the Primary Sensory Organ, Tongue:

Taste is primarily mediated by the endogenous gustatory organ, taste buds found within three of four types of papillae: fungiform, foliate, and circumvallate. The main muscular structure of the tongue is enclosed by an epithelial cell layer that contains small projections called papillae. Some regions of the papillae are specifically devoted to the detection of taste molecules and are called taste papillae or taste epithelium. The rest of the epithelium is non-gustatory epithelium covered with the fourth type of papillae, called filiform papillae. Filiform papillae are the most numerous of the papillae, but do not contain any taste buds. They are pyramidal in shape and contain a narrow tail of cornified cells that extend from the apical tip as a pennant.



(A,B) Adapted from Copyright © 2010 Pearson Education, Inc., publishing as Pearson Benjamin Cummings. (C) Adapted by permission from Macmillan Publishers Ltd: [NATURE] (Chandrashekar, J., Hoon, M. A., Ryba, N. J., & Zuker, C. S. The receptors and cells for mammalian taste. 444(7117), 288-294. doi: 10.1038/nature05401) copyright (2006).

Figure 1.2: Different components of the human taste system. Primary gustatory organ, tongue, taste papillae, and taste buds.

(A, B) Oral cavity is the primary gustatory surface and contains taste papillae at three different regions. Fungiform papillae are located in the anterior one third of the tongue, circumvallate papillae are located in the back of the tongue, and foliate papillae are located in the lateral posterior portion of the tongue. (C) Taste buds are composed of 50 to 150 taste sensory cells (Species dependent). A taste sensory cell extends microvilli/gustatory hair into the taste pore, a depression in the epithelium, where taste molecules can interact with taste cells. At the base, taste buds are innervated by nerve fibers.

Taste papillae: The fungiform papillae located in the anterior third of the tongue are named for their mushroom shaped structure consisting of a slender neck and an enlarged head. Fungiform papillae are generally not continuous, and are interspaced by filiform papillae. A fungiform papilla generally only contains a few taste buds. Foliate papillae at the lateral posterior portion of the tongue consist of parallel rows of folia and valleys and can contain hundreds of taste buds. At the posterior region of the tongue are the circumvallate papillae which, together, form a V-shaped line across the root of the tongue in humans. The number of taste buds in circumvallate papillae varies across

different species. Circumvallate papillae in human can contain thousands of taste buds. (Roper, 1992; Kim et al., 2003; Breslin & Huang, 2006) (Figure 1.2A, B).

Taste Buds: In mammals, the taste buds were first described in 1867 by Schwalbe and London (Witt, Reutter, & Miller, 2003). There are around 5000 taste buds in the oral cavity in human (Miller, 1995). Typically a taste bud is about 20-40 mm in diameter and 40-60 mm in length and is embedded in the surrounding scarified epithelium of the oral cavity (Chaudhari & Roper, 2010). The onion or garlic clove appearance of a taste bud comes from the side-by-side packing of elongated polarized taste cells. The opening of a taste bud on the surface of the tongue is called the taste pore where the apical end of the TSCs can come in contact with the environment (Farbman, Hellekant, & Nelson, 1985; Roper, 1992; Northcutt, Plassmann, Holmes, & Saidel, 2004; Breslin & Huang 2006) (Figure 1.2C).

Each taste bud houses a cluster of up to 50-150 bipolar neuroepithelial TSCs. Again the number of taste cells in a taste bud varies across species. The elongated taste sensory cells are mature differentiated cells and are surrounded by a layer of keratinocytes and basal cells (Kinnamon & Roper, 1987; Stone, Tan, Tam, & Finger, 2002; Finger, 2005). The basal cells that differentiate into TSCs are also known as Type IV taste cells. The apical tip of a TSC contains microvilli that extends into the taste pore and continuously samples the environment. Thus taste cells continuously experience wide fluctuations of tonicity, osmolarity and may come in contact with potentially toxic compounds. Therefore, similar to olfactory system, the TSCs are a population of continuously renewing cells with an average life span of 8-12 days (Beidler & Smallman,

1965; Farbman, 1980; R. J. Delay et al., 1986; Stone, Finger, Tam, & Tan, 1995; Okubo, Clark, & Hogan, 2009). The basal cells in the taste buds are also called progenitor cells that are believed to produce transit amplifying cells through asymmetrical division (Farbman, 1980). Subsequently, these transit amplifying cells gives rise to the specialized TSCs (Beidler & Smallman, 1965; Farbman, 1980; R. J. Delay, Kinnamon, & Roper, 1986; Miura, Kusakabe, & Harada, 2006).

1.2.2. Taste Bud Cells:

TSCs are designated into three categories, (1) Type I, (2) Type II, and (3) Type III cells and can be distinguished by their ultrastructure and specific markers (Murray, 1993; Pumplin et al., 1997; Yee, Yang, Bottger, Finger, & Kinnamon, 2001; DeFazio et al., 2006; Romanov & Kolesnikov, 2006). Early electron micrographs of taste buds revealed cells of varying density. Detailed morphological analysis of the ultrastructure of the cells demonstrated different cell types in the taste buds (R. J. Delay et al., 1986). Later, identification of specific protein markers associated with specific cell types concluded cell types with distinct functions (Figure 1.3).

Type I Cells: Type I taste cells are most abundant in number but least understood. Ultrastructural studies of taste buds show that these cells have a spindle shaped structure, contain dark granules, and irregularly shaped nucleus. These cells are characterized by numerous cytoplasmic processes that wrap other cells, thus are thought to have a glial-like function (Finger, 2005). Type I cells extend a microvillus into the taste pore where taste molecules can come in contact with the cell.

Type I cells express specific marker proteins like an ecto-ATPase NTPDase 2 and a glutamate aspartate transporter or GLAST (Pumplin, Getschman, Boughter, Yu, & Smith, 1999; Bartel, Sullivan, Lavoie, Sevigny, & Finger, 2006). Both of these proteins are involved in terminating neurotransmitter action, hence further supports the glial-like function of Type I cells. Furthermore, patch clamp studies suggest that Type I cells express ROMK channels that may function in K^+ homeostasis in the taste buds (Bartel et al., 2006; Dvoryanchikov, Sinclair, Perea-Martinez, Wang, & Chaudhari, 2009). During extended trains of action potentials elicited by strong taste stimuli, Type I cells may help in eliminating K^+ ions from extracellular space, thus serving another glial-like function. Recent finding suggests that a subtype of Type I cell may express functional epithelial sodium channel (ENaC), which is probably involved in salt taste transduction (Vandenbeuch, Clapp, & Kinnamon, 2008) (Figure 1.3A).

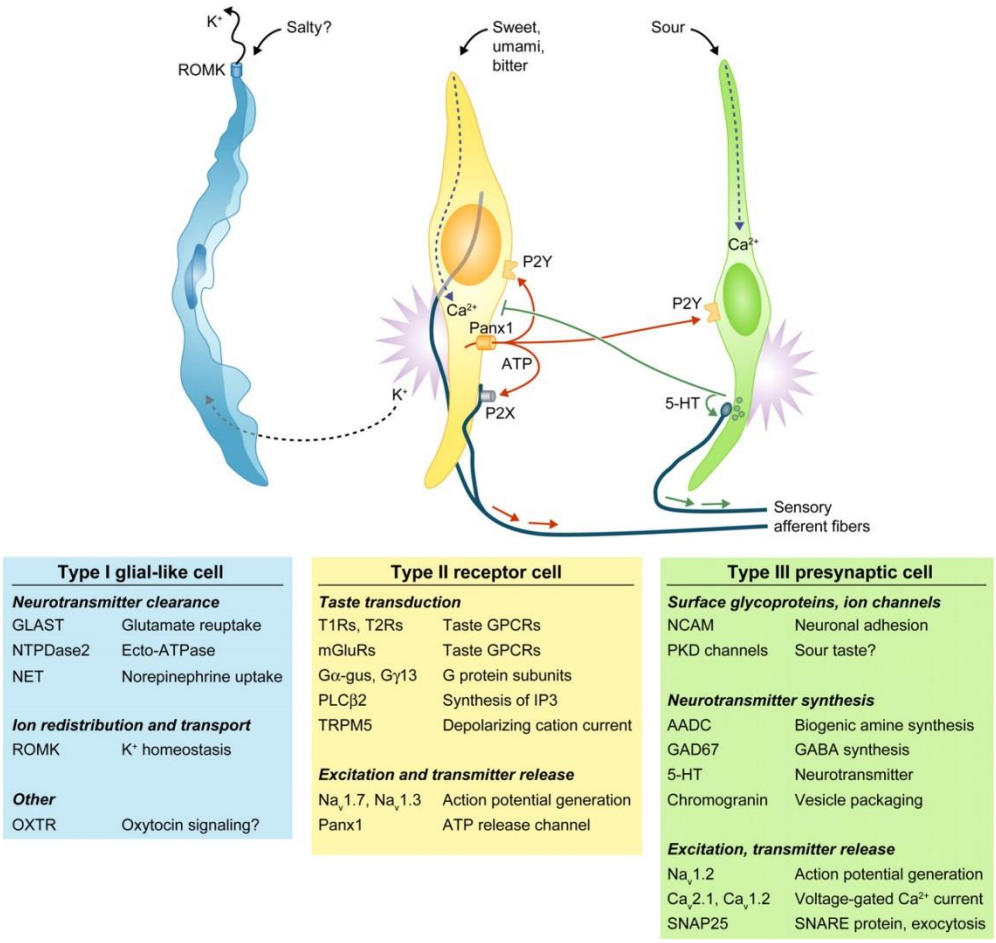


Figure 1.3: Type I, Type II, and Type III TSCs.
Functional classification the taste sensory cells are primarily based on the expression of specific proteins in each cell. Type I cells are also called glial-like cells because they perform many glial like functions, like removal of neurotransmitters released by Type II (ATP) and Type III (Serotonin (5-HT), and Norepinephrine (NE)) cells from extracellular space, K^+ homeostasis etc. These cells also express Na^+ channels and are proposed to be involved in the detection of salt taste. Type II cells also termed as receptor cells because they express receptors for sweet, bitter, and umami compound. Activation the Type II cells causes release of ATP, which then activates afferent nerve fibers or adjacent Type III cells. Additionally ATP also affects Type II cells in a positive feedback pathway. Type III cells are also called pre-synaptic cells because they form classical synapses with afferent nerves. Activation of type III cells causes release of neurotransmitter, 5-HT, which then activated afferent nerve fibers. Additionally serotonin is also proposed to have an inhibitory effect on Type II cells. Type III cells also express PKD channels and are thought to mediate sour taste.

Type II Cells: Type II cells are also called light cells because they are relatively electron transparent. These cells have round oval nuclei and an elongated structure, and they are mainly located at the periphery of the taste buds and are often found at the upper

part of a taste bud. In addition, they do not always extend to the basal part of the taste buds. These cells contain several short microvilli that extend into the taste pore. Type II cells are also called receptor cells because they express receptors for sweet, bitter, and umami compounds (DeFazio et al., 2006). Several studies suggest that the receptors for each of the taste quality are expressed in mutually exclusive fashion, thus forming three function subsets of sweet sensitive, bitter sensitive, and umami sensitive Type II cells (G. Nelson et al., 2001; Zhang et al., 2003; Tomchik, Berg, Kim, Chaudhari, & Roper, 2007). However, some physiological and molecular studies suggest that at least in a subset of type II cells, receptors of different taste qualities may be expressed in the same cell (Kusuhara et al., 2013).

The taste receptors expressed in Type II cells are G-protein coupled receptors, such as Taste receptor type 1 (T1R) and Taste receptor type 2 (T2R) families of receptors (Hoon et al., 1999; Miyoshi et al., 2001) and metabotropic glutamate receptors (mGluRs) (Chaudhari et al., 1996, Chaudhari, Landin, & Roper, 2000; Yamaguchi & Ninomiya, 2000; Toyono et al., 2002, 2003; San Gabriel, Uneyama, Yoshi, & Tori, 2005). Details about the involvement of specific receptors with specific taste stimuli are described in the section on transduction mechanisms. In addition to receptors, Type II cells also express all the components of the IP_3 second messenger system that includes G-proteins α-gustducin and transducin, (Yang, Tabata, Crowley, Margolskee, & Kinnamon, 2000; Margolskee, 2002; He et al., 2004), β_1 or $\beta_{3,}\gamma_{13,}$ phospholipase C (PLCβ2) (Clapp, Stone, Margolskee, & Kinnamon, 2001), inositol triphosphate receptor type III (IP_3R3) (Clapp et al., 2001), transient receptor potential channel 5 (TRPM5) (Clapp, Medler, Damak,

Margolskee, & Kinnamon, 2006), and pannexin hemichannels (Panx1) (Chandrasekhar et al., 2006; Romanov et al., 2007). Knockout mice lacking these second messenger components, such as α -gustducin, PLCβ2, IP3R3, or TRPM5 showed strongly diminished responses to taste stimuli (H. Xu et al., 2004).

Activation of the Type II taste cells by taste stimuli causes release of ATP. Interestingly, these cells do not form conventional synapses. Instead, they are thought to communicate with Type III cells or with the closely opposed nerve fibers via the release of ATP (Yang, Crowley, Rock, & Kinnamon, 2000; Yee et al., 2001; Clapp, Yang, Stoick, Kinnamon, & Kinnamon, 2004) (Figure 1.3B).

Type III Cells: Type III cells exhibit intermediate electron density compared to Type I (high) and Type II (low) cells. These cells contain a single long, slender microvillus that extends into the taste pore. Additionally, Type III cells also express neural and synaptic proteins such as neural cell adhesion protein (NCAM), and synaptosomal associated protein (SNAP 25) (Finger, 2005; Defazio et al., 2006). These cells are also called pre-synaptic cells because they form conventional synapses with nerve fibers and show presynaptic specialization (Clapp et al., 2006). They do not respond to sweet, bitter, or umami compounds, but express voltage-gated calcium channels (VGCC), and thus can be activated by depolarizing concentrations of extracellular K^+. Although activation of TSCs by depolarizing concentrations of extracellular K^+ has been used to distinguish between Type II and Type III cells, some recent studies reported that a subset of Type II cells can be activated by high K^+ solution (Gilbertson, Fontenot, Liu, Zhang, & Monroe, 1997; Hacker, Laskowski, Feng, Restrepo,

& Medler, 2008), thus suggesting that VGCC is expressed in a subset of Type II cells. Nevertheless, activation of Type III cells causes release of serotonin (5-HT) and to a lesser extent norepinephrine (NE) (Defazio et al., 2006; Y. A. Huang, Maruyama, & Roper, 2008) (Figure 1.3C).

Type III cells also express certain members of the transient receptor potential family protein, PKD2L1 and PKD1L3 (A. L. Huang et al., 2006; Ishimaru et al., 2006; Lopez Jimenez et al., 2006). Cation channels formed by these proteins are thought to be involved in sour taste detection. These channels are sensitive to drops in extracellular pH (sour stimulus) (DeSimone, Lyall, Heck, & Feldman, 2001; Lyall et al., 2001; Y. A. Huang, Maruyama, Stimac, & Roper, 2008). Moreover, mice lacking PKD2L1 also do not show cranial nerve responses to acidic taste stimuli, thereby suggesting a role for PKD2L1 in sour taste transduction (A. L. Huang et al., 2006). However mice lacking PKD1L3 can detect sour taste stimuli (T. M. Nelson et al., 2010).

Type III cells also express certain plasma membrane potassium channels that can also serve as plausible candidate sour detectors (Lin, Burks, Hansen, Kinnamon, & Gilbertson, 2004; Richter, Dvoryanchikov, Chaudhari, & Roper, 2004). Moreover, Type III cells are also involved in the detection of carbonation (Graber & Kelleher, 1988; Simons et al., 1999; Chandrashekar et al., 2009). Genetic knockout studies have identified carbonic anhydrase 4, an enzyme attached to the cell surface of Type III cells through a glycosylphosphatidylinositol anchor, which at least in part functions as a carbonation detector. Additionally, synaptic transmission in Type III taste cells is also

critical for taste responses to carbonation (Chandashekar et al., 2009). However, the complete transduction mechanism for sour or carbonation is not yet understood.

Type IV Cells: Type IV cells have a shape similar to that of epithelial cells in the stratified squamous epithelium, and are generally present at the base of the taste buds. Unlike the TSCs, Type IV cells do not possess any microvilli or reach to the taste pore. These cells are also known as progenitor cells for other cell types and express the developmental signaling protein sonic hedgehog (Miura et al., 2003) (Figure 1.3).

Perigemmal Cells: Another cell type, called perigemmal cells, can often be found in the periphery and in the taste buds. These cells also have a shape similar to that of Type IV cells and they also do not extend to the taste pore. Generally these cells are found in layers in the periphery of taste buds and form a network of keratin bundles. However when they extend into the taste bud they take the shape of Type I cells and are thought to form a diffusion barrier to restrict the access of small molecules by lateral diffusion through the surrounding epithelium (Sakai, Kaidoh, Morino, & Inoue, 1999).

1.3 Transduction Mechanisms of Taste Molecules by the TSCs

Among the five basic tastes, salt and sour tastes are detected by ion channels expressed in the apical end of the TSCs, and sweet, bitter, and umami tastes are detected by specific G-protein coupled receptors.

1.3.1 Salt Taste Transduction:

Salt taste is transduced by depolarization of the TSCs by Na^+ ions directly permeating through the ion channels located in the apical end of TSCs. Type I TSCs are thought to be involved in salt taste transduction (Vandenbeuch et al., 2008). Candidate

receptors for salt taste include an amiloride sensitive epithelial Na^+ channel, ENaC (Heck, Mierson, & DeSimone, 1984; Lin, Finger, Rossier, & Kinnamon, 1999; Lindenmann, 2001), an amiloride insensitive epithelial Na^+ channel (Ye et al., 1991), and a modified transient receptor potential V1 or TrpV1 channel (Ruiz, Gutknecht, Delay, & Kinnamon, 2006; Treesukosol, Lyall, Heck, DeSimone, & Spector, 2007).

Mice in which a critical EnaC subunit is knocked out show impaired salt detection, establishing its role in salt taste (Chandrashekar et al., 2010). Although a modified TrpV1 channel has been proposed in salt taste, TrpV1 knockout mice display minimal loss-of-function phenotype with respect to the detection of salt (Ruiz et al., 2006; Treesukosol, et al., 2007). A recent study with knockout mice suggests a role for calcium homeostasis modulator 1 (CALHM1) as an important component of the salt transduction pathway (Tordoff et al., 2014). However, the detailed pathway is not clear (Figure 1.4).

1.3.2 Sour Taste Transduction:

The main stimulus for sour taste is intracellular proton concentration. Sour taste is detected by presynaptic or type III cells (Y. A. Huang et al., 2008). Receptors or channels for sour transduction are not completely understood. PKD2L1 and PKD2L3 are proposed to be involved in sour transduction (A. L. Huang et al., 2006; Ishimaru et al., 2006). However some studies question the involvement of PKD2L1 and PKD2L3. Other suggested receptors for the sour taste include voltage dependent K^+ channels (Kinnamon, Dionne, & Beam, 1988; Ishimaru et al., 2006). Again, more investigation is required to fully understand the receptors and transduction mechanisms for sour taste (Figure 1.4).

1.3.3 Sweet, Bitter, and Umami Taste Transduction:

Each of these three taste qualities are detected by specific GPCRs and to some extent share the same transduction pathway. Here the receptors for each taste molecule are described first.

Sweet Taste Receptors: The heterodimer receptor T1r2+T1r3 is proposed to be the main receptor for detection of sugar, synthetic sweeteners, and sweet-tasting proteins such as monellin and brazzein (G. Nelson et al., 2001; Jiang et al., 2004; H. Xu et al., 2004). Co-expression of T1r2 and T1r3 in HEK cells and the activation of those cells by sweet compounds have provided strong evidence that the heterodimer constitutes the sweet receptor (G. Nelson et al., 2001). However, mice lacking T1r3 receptor can detect sweet taste and discriminate it from other taste qualities, suggesting involvement of multiple receptors in sweet taste (Damak et al., 2003; E. R. Delay, Hernandez, Bromley, & Margolskee, 2006). Interestingly, a single knockout of either T1r2 or T1r3 eliminates all behavioral preferences for artificial sweeteners (Damak et al., 2003; Zhao et al., 2003).

T1rs are class C GPCRs, which have a large N terminus venus fly trap (VFT) domain. Interestingly, different sweeteners bind to different domains or different subunits of the T1r2+T1r3 receptor. Natural sugars, as well as some artificial sweeteners (e.g., aspartame), bind in the Venus flytrap domain of T1r2. However, some ligands also bind to the transmembrane domain (example cyclamate and T1r1+T1r3 blocker lactisole). Additionally, the sweet proteins bind to the cysteine rich domains (Cui et al., 2006;

Temussi, 2012). These differences in receptor molecules might answer the question how taste qualities of artificial sweeteners and natural sugars can be distinguished.

Bitter Taste Receptors: In vertebrates, bitter compounds are detected by a family of T2r receptors. These receptors are structurally related to rhodopsin family and range in 3 to 49 in number depending on the species (Chandrashekar et al., 2000; Matsunami, Montmayeur, & Buck, 2000; Shi & Zhang, 2006). Generally bitter responsive cells express multiple bitter receptors, thus bitter molecules are sometimes difficult to distinguish by taste alone (Mueller et al., 2005). However, not all bitter receptors are expressed in the same cell; consequently there are possible subtypes of bitter sensitive TSCs (Weiss, Dahanukar, Kwon, Banerjee, & Carlson, 2011; Voigt et al., 2012). Knockout studies have further established the function of T2rs in bitter taste. For example, knocking out a single bitter receptor, T2r5, eliminated behavioral and nerve responses to the receptor agonist cycloheximide for the same concentrations that evokes strong bitter taste response in wild type mice (Mueller et al., 2005).

Bitter receptors can be categorized in two classes: 1) specialists that detect one or few specific bitter tasting compounds and 2) generalists that detect many different compounds (Behrens & Meyerhof, 2009). Although the generalists can detect many compounds, they are less sensitive in comparison to the specialist group. Recent findings show that naturally occurring compounds such as sesquiterpene lactones from plants, can function as an agonist at one bitter receptor and as an antagonist at another bitter receptor, which adds further complexity to the taste receptor system (Brockhoff et al., 2011; Liman, Zhang, & Montell, 2014).

Umami and L-amino acid Receptors: A heterodimer of the T1r receptor family, T1r1+T1r3, is widely recognized as the umami receptor. In HEK cell expression studies, mouse T1r1+T1r3 responds to a large array of L-amino acids, sometimes only in the presence of nucleotides. However, human T1R1+T1R3 respond only to umami L-amino acids. Additionally, the receptor from both species recapitulates the special characteristic of the umami response, specifically response potentiation of glutamate by IMP and GMP. It is suggested that IMP and GMP are not agonists of the heterodimer but rather bind and stabilize the receptor in the glutamate-bound state (Zhang et al., 2008). However, human and mice both can detect IMP and GMP individually thus suggesting additional receptors for umami taste. Similar to the study with sweet receptors, in mice the genetic disruption of umami T1rs has produced controversial results. Even though one research group found a complete loss of umami taste in T1r1 and T1r3 knockout mice (Zhao et al., 2003), other groups with independently developed knockout mice found only reduced taste for umami and L-amino acids, suggesting there are multiple receptors in umami taste (Damak et al., 2003; E. R. Delay, Hernandez, Bromley, & Margolskee, 2006; Kusuhara et al., 2013; Pal Choudhuri, Delay, & Delay, 2015). While these differences can be atributed to the difference in the production of knockout models, there is increasing evidence suggesting multiple receptors for umami and L-amino acid taste. Additionally, there is strong evidence that suggests a role of mGluRs in umami taste (See section 1.6 for detailed discussion on Umami and L-amino acid taste).

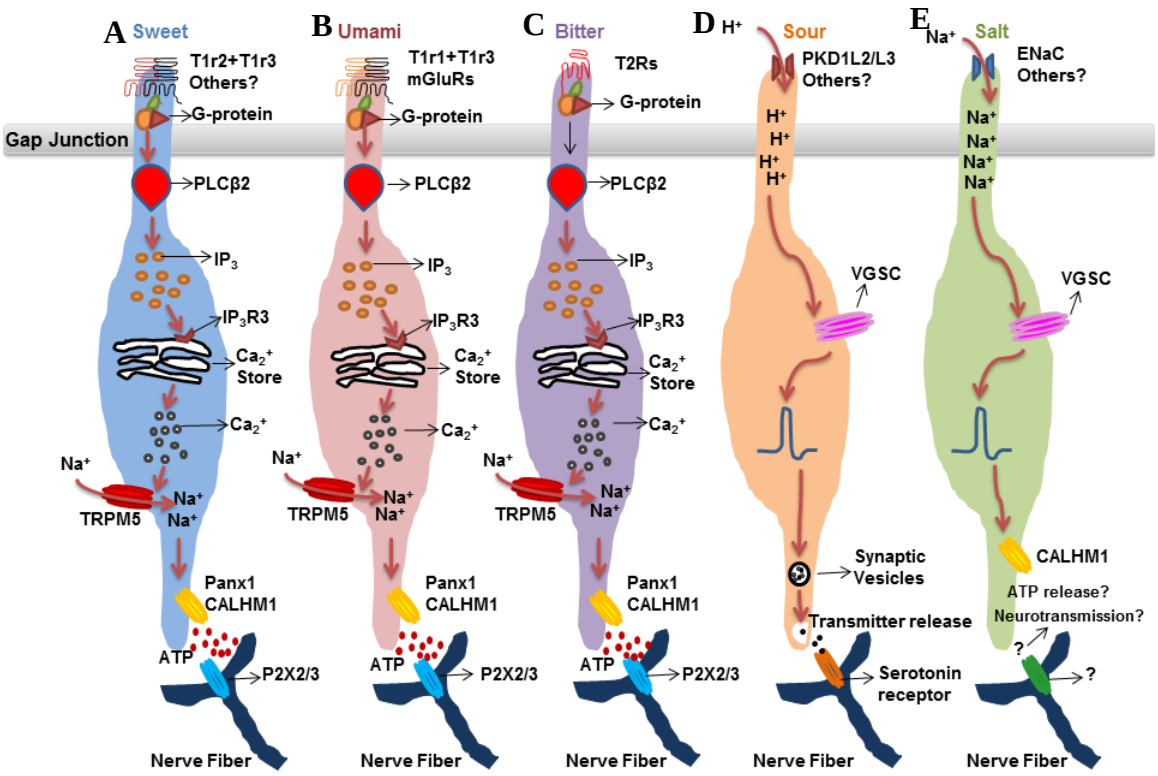


Figure 1.4: Transduction mechanisms for sweet, umami, bitter, sour, and salt taste.
(A, B, C) Sweet, umami, and bitter compound are detected by specific GPCRs expressed at the apical end of the TSC. Activation of the receptors activates associated G-protein which then in turn activates PLCβ2. This breaks down PIP2 and produces cytosolic IP_3. IP_3 then binds to IP_3R3 receptors on endoplasmic reticulum and causes Ca^{2+} release. The Ca^{2+} can have multiple functions. It activates cation channel TRPM5. Influx of Na^+ ions through TRPM5 depolarizes the cells and probably causes firing of action potentials. This, along with Ca^{2+}, probably opens the hemichannel pannexin 1 and the Ca^{2+}channel CALHM1 and causes ATP release. Released ATP activates afferent nerve fibers and/or Type III cells. (D, E) Sour and salt tastes are mediated by ion channels expressed in the apical end of TSCs. But the pathways are not known in much detail.

Sweet, Bitter, and Umami/L-Amino Acid Transduction: Two major streams of intracellular molecular signal cascade appear to be activated by taste stimulation of specific taste GPCRs by sweet, bitter, and umami compound. The earliest identified taste transduction mechanism involved the second messenger cyclic adenosine monophosphate (cAMP). Later the PLCβ2 mediated pathway was identified. However at this point

PLCβ2 mediated pathway is better understood compared to the cAMP mediated one. Thus we will first discuss the PLCβ2 pathway (Figure 1.4).

PLC mediated Pathway: Sweet, bitter, and umami tastes are mediated by a PLC mediated pathway (Zhang et al., 2003). In this pathway, activation of a specific receptor causes activation of the associated G-protein and a resulting dissociation between the α subunit and the βγ subunit. This βγ subunit activates the membrane bound enzyme PLCβ2, which in turn breaks down PIP_2 and produces cytosolic IP_3 and membrane bound DAG. The IP_3 binds to IP_3R3 receptor in the endoplasmic reticulum and causes the release of calcium (Ca^{2+}) from intracellular stores. The Ca^{2+} binds to and activates the non-selective cation channel TRPM5. Influx of positive ions, especially Na^{+}, causes depolarization. Support for this model comes from the observations that inactivating mutations of PLCβ2, IP_3R3, or TRPM5 severely diminish behavioral responses to bitter, sweet, and umami taste (Zhang et al., 2003; Damak et al., 2006; Tordoff & Ellis, 2013). It is generally assumed that tastes of all L-amino acids are also transduced in the same pathway. However, no experiment has been conducted to support this hypothesis.

How depolarization of the cells finally causes the release of ATP is not entirely understood. ATP is thought to be released through Panx1 hemichannels (Huang et al., 2007; Romanov et al., 2007; Murata et al., 2010). Panx1 is robustly expressed in receptor cells and are gated, opened by elevated cytoplasmic Ca^{2+} and/or membrane depolarization (Locovei, Wang, & Dahl, 2006). In addition, Panx1 selective antagonists can block taste mediated ATP release (Y. J. Huang et al., 2007; Dando & Roper, 2009).

A recent study suggests that ATP release is also mediated by CALMH1 (Taruno, Matsumoto, Ma, Marambaud, & Foskett, 2013; Taruno, Vingtdeux, et al., 2013), a calcium sensing ion channel. This channel is highly enriched in TRPM5-expressing cells and can release ATP in heterologous cells. Furthermore, CALMH1 knockout animals exhibit diminished abilities to detect the tastes of bitter, sweet, and umami compounds (Taruno et al., 2013; Taruno, Vingtdeux, et al., 2013). Whether CALMH1 acts alone or in combination with panx1 remains to be determined.

Although PLCβ2 mediated pathway have been well established in sweet, bitter, and umami detection, recent studies also suggest activation of alternate pathways that are independent of PLCβ2 and TRPM5 mediated pathways (Hacker et al., 2008). Knocking out PLCβ2 severely diminishes but does not completely eliminate taste sensitivity (Zhang et al., 2003; Dotson, Roper, & Spector, 2005). Hacker et al., (2008) found that taste cells lacking PLCβ2 can be activated by bitter stimuli, thus indicating another signal pathway exists. Further, immunohistochemical identification of PLCβ3 and IP_3R1 on taste cells indicated presence of multiple PLC mediated pathways (Hacker et al., 2008) in TSCs.

cAMP Mediated Pathway: The cAMP pathway was first studied in reference to the sweet taste. In 1989, Striem et al. demonstrated that the treatment of tongue epithelium from the anterior portion of rat tongue by sucrose caused an increase in cAMP level, suggesting activation of $G\alpha_s$ mediated activation of adenylyl cyclase pathway (Striem, Pace, Zehavi, Naim, & Lancet, 1989). Later reports showed that activation of taste epithelium by sweet stimuli also yielded the same results (Trubey, Culpepper, Maruyama, Kinnamon, & Chaudhari, 2006).

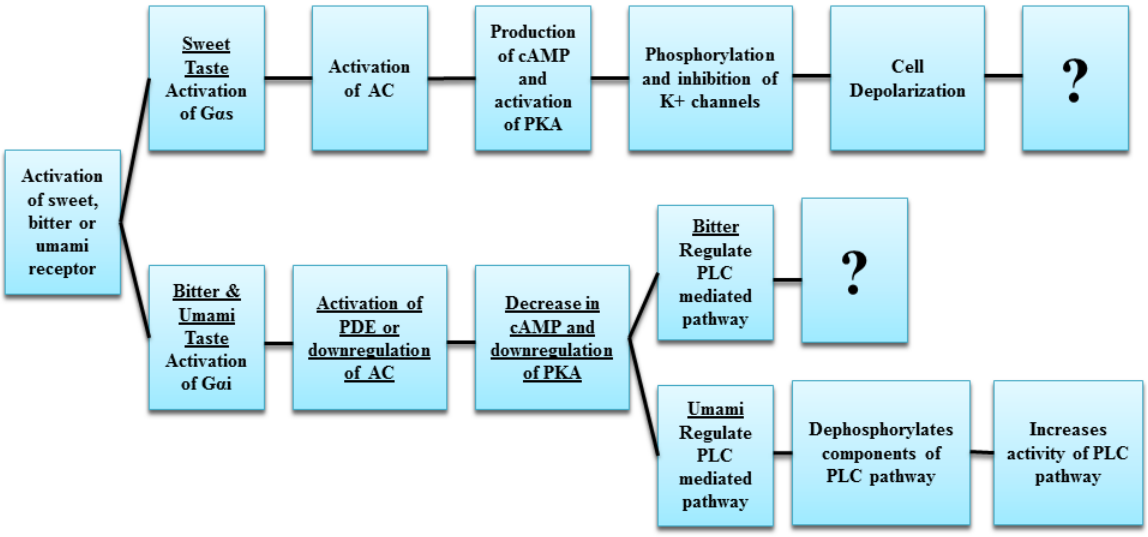


Figure 1.5: cAMP mediated pathway for sweet, bitter, and umami taste transduction.
The pathway is not completely understood. AC, Adenylyl cyclase; PDE, Phosphodiesterase; PKA, protein kinase A.

The first identified component of the taste signaling pathway was a specific Gα subunit, termed gustducin (McLaughlin, McKinnon, & Margolskee, 1992; Wong, Gannon, & Margolskee, 1996). Gustducin is very closely related to transducin and is a member of the $G\alpha_i$ family (McLaughlin, McKinnon, Spickofsky, Danho, & Margolskee, 1994; Hoon, Northup, Margolskee, & Ryba, 1995). Contrary to the association of sweet taste with $G\alpha_{s,}$ transduction of the bitter and umami tastes are proposed to be associated with $G\alpha_{i.}$ Direct measurement of the cAMP level in taste epithelium after stimulation with bitter or umami taste stimulus revealed a decrease in cAMP level (Yan et al., 2001; Trubey, Culpepper, Maruyama, Kinnamon, & Chaudhari, 2006). The pathway downstream of cAMP is not clear.

In the case of sweet taste, activation of cAMP causes activation of protein kinase A (PKA), which in turn is proposed to phosphorylate and block K^+ channels resulting in

cell depolarization (Avenet, Hofmann, & Lindemann, 1988a, 1988b). This is thought to cause the release of neurotransmitter. In contrast, activation of TSCs by bitter or sweet stimuli is proposed to activate gustducin or transducin (He et al., 2004). Both of these can either activate phosphodiesterase or downregulate adenylyl cyclase (Wong, Gannon, & Margolskee, 1996; Abaffy et al., 2003), which breaks down cAMP and thus decreases the amount PKA (McLaughlin et al., 1994). How this is involved in the bitter transduction is not clear but for umami, this decrease in cAMP was proposed to play an important role in synergistic responses (Kinnamon, Lin, Ogura, Ruiz, & Delay, 2005; Kinnamon, 2009; Kinnamon & Vandenbeuch, 2009). According to this hypothesis, down regulation of PKA causes the release of phosphorylation from the components of the PLC mediated pathway, thereby enabling further activation of the PLC pathway in synergistic responses (Figure 1.5).

1.3.4 Cell-Cell Communication in Taste Buds:

Activation of Type II cells by taste stimuli causes the release of ATP through pannexin hemichannels and/or CALHM1 channels (Y. J. Huang et al., 2007; Dando & Roper, 2009; Murata et al., 2010; Taruno et al., 2013; Taruno, Vingtdeux, et al., 2013). The released ATP or sour stimuli can activate Type III cells and cause release of different neurotransmitters such as serotonin (5-HT) and norepinephrine (NE) via Ca^{2+}-dependent exocytosis. In some instances NE is co-released with 5-HT (Dvoryanchikov, Tomchik, & Chaudhari, 2007; Y. A. Huang et al., 2008).

Calcium imaging experiments of TSCs showed that the ATP released by Type II taste cells acts as a paracrine as well as an autocrine signaling molecule. The ATP

activates gustatory nerve fibers and Type III cells by binding to the P2X (on nerve fibers) or P2Y (Type III cells) receptors and also binds to purinergic receptors (P2Y) on Type II cells and promotes further release of ATP, thus producing a positive feedback signal (Y. A. Huang, Dando, & Roper, 2009). The ecto-ATPase released by Type I cells, breaks down ATP and terminates the signals in taste buds.

5-HT released by Type III cells acts on multiple targets. 5-HT exerts a negative feedback signal on Type II cells and inhibits Type II cells (Y. A. Huang et al., 2009). Additionally, GABA is proposed to function as an inhibitory neurotransmitter in taste buds and may modulate taste responses (Obata, Shimada, Sakai, & Saito, 1997; Cao, Zhao, Kolli, Hivley, & Herness, 2009; Y. A. Huang, Pereira, & Roper, 2011). Some recent studies also suggest a role for glutamate as a neurotransmitter in the taste buds, but to date there is no direct evidence that supports this hypothesis (Caicedo, Jafri, & Roper, 2000; Vandenbeuch et al., 2010)

1.4 Taste Signaling from Tongue to Brain

Non-taste components of the tongue in general are innervated by fibers of three cranial nerves (CN) (Witt et al., 2003). These include: 1) motor nerve fibers arising from the hypoglossal nerve (CN XII), 2) somatosensory nerve fibers projecting through the mandibular division of the trigeminal (CN V3) and glossopharyngeal (CN IX) nerves, and 3) autonomic nerve fibers stemming from inter-medio facial nerve (CN VII), the glossopharyngeal (CN IX) nerve, and the vagus nerve (CN X).

Taste buds are innervated by afferent fibers of three cranial nerves. Taste buds of the fungiform papillae are innervated by the chorda tympani (CT) branch of CN VII.

Taste buds of the foliate and circumvallate papillae are innervated by lingual-tonsillar branch of CN IX. Taste buds in the pharyngeal and laryngeal epithelium are innervated by CN X. Thus, taste signals are mainly transmitted by the glossopharyngeal, chorda tympani and vagus nerves. Cell bodies of all these fibers are located in three peripheral ganglia, the geniculate ganglion (CN VII), the petrosal (XN IX), and the nodose ganglion (CN X). In adult animals, each taste bud is innervated by 3-14 sensory ganglion neurons (Whitehead et al., 1999). The fibers of ganglionic neurons terminate in a small region in the medulla called the gustatory nucleus, or the nucleus of solitary tract (NTS) (Whitehead & Finger, 2008). Information from NTS is transmitted in a descending pathway to various oromotor nuclei and salivatory nuclei of the brain stem which also governs gag reflexes, jaw movement, and salivation (Whitehead & Finger, 2008). This further suggests that taste information can also be important in governing various physiological functions such as salivation, jaw movement, control chewing, swallowing, opening of the mouth, etc (Roper, 2009).

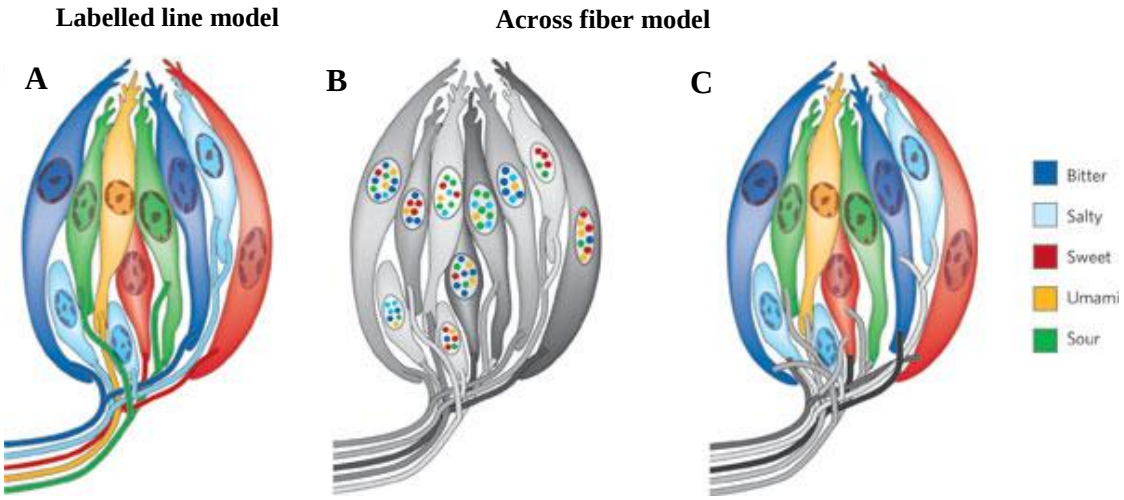
The projection neurons in the NST send their axons dorsally in the ascending lemniscal tract. Signals in this portion of the ascending lemniscal pathway are proposed to be important for the detection and discrimination of taste stimuli (Roper, 2009; Spector & Glendenning, 2009). In rodents this ascending pathway is relayed in the parabrachial nucleus of the pons. However, in humans and other primates, it connects directly to the relay neurons in the parvocellular division of the ventroposteromedial thalamic nucleus, or taste thalamic nucleus (Whitehead & Finger, 2008). Taste information from this nucleus then goes to a region of cerebral cortex, known as gustatory cortex. Human

neuroimaging studies show that a small number of neurons in the gustatory cortex represent taste quality (Schoenfield et al., 2004). From gustatory cortex, taste signals are transmitted to the secondary gustatory cortex, also known as orbifrontal cortex. Sensory modalities of taste, vision, and olfaction are proposed to converge in this region and construct the perception of flavor. Neurons of the secondary gustatory cortex are also proposed to be involved in satiety. Taste signal from orbito-frontal cortex goes to the amygdala and the lateral hypothalamus, which is proposed to impart hedonic value to taste sensations and integrate taste information in the context of energy need and reward (Hofmann et al., 2011). Finally, cortical taste areas send afferents to the NTS/parabrachial nucleus allowing top-down modulation of gustatory processing at the level of the brainstem (Roper, 2006).

1.4.1 Neural Coding of Taste:

How the activation of TSCs gets translated into a neural code that specifies different taste qualities remains unclear. There are two possible hypotheses that have been under constant debate for a long time in the field of taste. These are the labelled line model and the across fiber model (Chandrashekar, Hoon, Ryba, & Zuker, 2006).

The labeled line model suggests nerve fibers are narrowly tuned, where particular TSC responds to single taste quality and a dedicated nerve fiber carries the information about only a single taste quality. The across fiber theory argues that the fibers are broadly tuned. According to this model, a single TSC can respond to a single or multiple taste qualities and a particular fiber can also carry information about multiple taste qualities.



Adapted by permission from Macmillan Publishers Ltd: [NATURE] (Chandrashekar, J., Hoon, M. A., Ryba, N. J., & Zuker, C. S. The receptors and cells for mammalian taste. 444(7117), 288-294. doi: 10.1038/nature05401) copyright (2006)

Figure 1.6: Taste coding: labelled line versus across fiber model.
(A) According to the labelled line model a single TSC detects only a specific taste quality, and single nerve fibers carry signals about only one particular taste quality. (B, C) The across fiber model has two variations. According to one variation (B), a single TSC can detect multiple taste qualities and thus each nerve fiber carries signals about multiple taste qualities. According to the other variation (C), TSCs are specific about taste qualities but a single nerve fiber carries information from multiple TSCs detecting different taste qualities.

Both models draw on electrophysiological data obtained from nerve recording studies. Modern evidence supporting the labeled-line model has been based on the results of genetically engineered mouse where a modified opioid receptor replaced sweet or bitter taste (Zhao et al, 2003; Mueller et al., 2005). The case in which the sweet receptor cell was modified, mice strongly preferred solutions of synthetic ligand for the modified opioid receptor, as if the compound tasted sweet. Along the same line, when the bitter receptor was modified, mice strongly avoided the synthetic ligand as they would do for a bitter taste stimulus. Even though this seems like strong evidence for the labeled line model, the logic behind the experiment needs a second thought. The change of the receptor in the system does not change the neural coding and thus this cannot be directly concluded as a labeled line model (Figure 1.6).

Evidence for the across fiber model comes from electrophysiological recordings from single afferent fibers or their parent sensory ganglion cells. While some neurons respond strongly to a single taste quality, it can also exhibit weaker responses to other taste qualities. These fibers are also named depending on their response preferences, such as, “S-best” (responds very strongly to sweet) or “M-best” (responds very strongly to umami) fibers. In addition, there are other fibers that respond to multiple taste qualities and thus are broadly tuned (Hellekant, Danilova, & Ninomiya, 1997; Hellekant, Ninomiya, & Danilova, 1997; Gilbertson, Boughter, Zhang, & Smith, 2001; Caicedo, Kim, & Roper, 2002; Frank, Lundy, & Contreras, 2008; Breza, Nikonov, & Contreras, 2010). In summary, there are nerve fibers that are broadly tuned and some fibers are somewhat narrowly tuned.

Another less discussed model suggests temporal coding of the taste signal underlies the selective detection of different stimuli. This model proposes that a specific taste quality is denoted by a timing pattern of action potentials similar to the system for auditory fibers (Katz, Nicolelis, & Simon, 2002; Katz, Simon, & Nicolelis, 2002). One possibility is that all three models exist and the final result is generated as a combined effect of all these models.

1.5 Taste Receptors in a Myriad of Other Tissues

Taste receptors, specifically T1r andT2r families of receptors, calcium sensors (CaSR) and some L-amino acid taste receptors such as GPRC6A, were first identified in taste epithelium, where they are activated by sweet, bitter, or umami stimuli. However, over the past few years, these receptors have been identified in a wide array of other tissue. In addition to the receptors, the components of second messenger signaling

cascades found in TSCs are also found in those T1r and T2r expressing non-oral tissues (Li, 2013).

In most of the tissue these receptors function as chemical sensors of nutrients or harmful substances. However, in many tissues the functions of these receptors are not completely understood. The role of the taste receptors in some of the tissue is detailed below. See Table 1.1 for a brief description of the extra-oral T1rs and T2rs expression, and their functions.

1.5.1 Taste Receptors in the Gut:

Taste receptors can be found all through the digestive system and are probably associated with diet-related diseases (Rozengurt, 2006; Egan & Margolskee, 2008). In 1996, Hofer, Puschel, and Drenckhahn found gustducin expression in the brush cells of the stomach and the intestine. Later, T1Rs and T2Rs were also discovered in the guts of rodents and humans (Raybould, 1998). In the stomach, these receptors and the transduction components are co-localized in ghrelin (an appetite-inducing peptide) releasing gastric secretory cells. Although it is not clear if the activation of these receptors by the chemicals in the food directly regulate ghrelin release, there are, however, strong lines of evidence that ghrelin levels are modulated by ingestion of dietary substances (Hass, Schwarzenbacher, & Breer, 2007; Foster-Schubert, 2008; Hass, Schwarzenbacher, & Breer, 2010). In the distal stomach, L-amino acids can modulate the secretion of gastrin and somatostatin (Feng et al., 2010; D. C. Haid, Jordan-Biegger, Widmayer, & Breer, 2012). These cells express L-amino acid receptors GPRC6A and CaSR, but not the T1r3 (Feng et al., 2010; D. C. Haid et al., 2012; D. Haid et al., 2013)

In the small intestine, gustducin is expressed various subtypes of enteroendocrine cells. These include K, L, and K/L cells. These cells release the gut hormones glucagon-like peptide-1 (GLP-1) and/or glucose-dependent insulinotropic peptide or the gastric inhibitory peptide. The T1r2+T1r3 is selectively expressed in the L cells, which releases the peptide GLP-1 upon sugar ingestion. GLP-1 then increases the release of insulin from the pancreas (Mace, Affleck, Patel, & Kellett, 2007; Margolskee et al., 2007; Kokrashvili, Mosinger, & Margolskee, 2009).

L-amino acids receptors also stimulate secretion of satiation hormone cholecystokinin (CCK) (Ballinger & Clark, 1994a, b). Similarly, bitter tasting compounds can also induce release of another hormone from an enteroendocrine cell line (STC-1 cells) by affecting Ca^{2+} influx (Wu et al., 2002; Chen, Wu, Reeve, & Rozengurt, 2006; Jeon, Zhu, Larson, & Osborne, 2008). This is proposed to reduce gut motility (Masuho, Tateyama, & Saitoh, 2005). Activation of T2Rs by bitter compounds in the STC-1 cells release the peptide hormone cholecystokinin (CCK), which is proposed to reduce gut motility (Masuho et al., 2005)

It is important to note that there are also several receptors in the gut that detect fatty acids and may modulate hormonal secretions (Edfalk, Steneberg, & Edlund, 2008; Liou et al., 2011). However, we limited our discussion to the receptors for primary taste qualities.

1.5.2 T1rs and T2rs in the Airway Cells:

T1rs, T2rs, and their taste transduction cascade have been found throughout the airways (Tizzano et al., 2010; Tizzano, Cristofoletti, Sbarbati, & Finger, 2011; Pulkkinen,

Manson, Safholm, Adner, & Dahlen, 2012). Interestingly, T2rs are present on the cilia of the epithelial cells (A. S. Shah, Ben-Shahar, Moninger, Kline, & Welsh, 2009), where they can detect environmental toxins. Recently, several studies reported that the smooth muscle cells of the human airways co-express T2Rs with gustducin and some components of the PLC-mediated taste signaling cascade (Deshpande et al., 2010; Doggrell, 2011). The distribution of taste receptors in the airway are a defensive mechanism against the inhalation of irritants (Tizzano et al., 2010). The T2R bitter taste receptor in the human upper airway cilia is thought to detect bacterial molecules and stimulate nitric oxide (NO) production (Waterer, 2012). NO production is instantaneous and causes increases in ciliary beat frequency (A. S. Shah et al., 2009). NO can also directly diffuse into the airway surface liquid, where it exhorts bactericidal effect. This way T2Rs participate in innate immunity. Curiously, activation of T2Rs by bitter compounds increases intracellular Ca^{2+} and causes relaxation of airway smooth muscle cells rather than contraction of these muscles by opening calcium-activated big potassium (BKCa) channels (Deshpande et al., 2010).

1.5.3 T1R2+T1R3 Receptors in the Bladder:

Immunohistochemical studies have revealed the presence of T1R2+T1R3 in the plasma membrane of three urothelial cell types, particularly umbrella cells from human and rat bladders. Stimulation of the receptor by the artificial sweetener, sodium saccharin, can strengthen rat bladder smooth muscle contraction, suggesting a plausible physiological function of T1R2+T1R3 in the urinary bladder (Elliott, Kapoor, & Tincello, 2011).

1.5.4 Taste Receptors in the Brain:

Immunostaining and in situ hybridization analysis have revealed that T1r1, T1r2, and T1r3 are expressed in the mammalian brain along with their associated G-proteins. Particular regions include the hypothalamus, hippocampus and cortex. Interestingly, the expression of T1rs and T2rs has been detected in both neurons and non-neuronal cells. Therefore, the sweet receptor, T1r2+T1r3 is thought to function as a general glucose sensor (Ren et al., 2009). Another study has revealed that neurons in the pyramidal cell and granule cell layers constitutively express the sweet receptor T1R2+T1R3 and α-gustducin. In addition, activated astrocytes in ischaemic hippocampi display up-regulated expression of taste receptor genes (Shin et al., 2010).

Since T1Rs are expressed in the gut and brain, these receptors are assumed to be related to glucose sensing, feeding and obesity (Egan & Margolskee, 2008). However T1r2- and T1r3-knockout mice show normal chow and fluid intake, body weight, and consume similar amounts of polycose as wild type mice (Treesukosol, Smith, & Spector, 2011a, 2011b).

In addition to the above mentioned tissue types, these taste receptors are also found in breast tissue and spermatozoa. However, their function in these cells is not completely understood. Altogether, the taste receptors in various non-oral regions are exhibiting emerging roles in health and disease and can be potential target for many disease conditions.

Table1.1: Expression and function of taste receptors in non-gustatory organ.

Organ/ Tissue	Cell Type Or Region	Expressed Taste Receptors	Physiological Role	Endogenous Ligand(s)	References
Airway (nose and sinuses)	Ciliated epithelial cells	T2R38 bitter receptors (human)	Nitric oxide production to increase cilia beating and directly kill bacteria	Bacterial AHL quorum-sensing molecules	Lee et al., 2012 Wilson, Wiens, & Smith, 2013
Airway (nose and sinuses)	Solitary chemosensory cells (SCCs)	Various T2R bitter receptors (mouse and human)	Antimicrobial peptide secretion (human); breath-holding and inflammation (mouse)	Unknown (human); bacterial AHL quorum-sensing molecules (mouse)	Finger et al., 2003; Gulbransen, Clapp, Finger, & Kinnamon, 2008; Tizzano et al., 2010.; Barham et al., 2013; Lee et al., 2014
		T1R2/3 sweet receptor (mouse and human)	Attenuate antimicrobial secretion (human); unknown (mouse)	Airway surface liquid glucose	Barham et al., 2013
Airway (trachea)	Chemosensory brush cells	Various T2R bitter receptors (mouse)	Breath-holding mediated by Ach release and trigeminal neuron activation (mouse); unknown (human)	Bacterial AHL quorum-sensing molecules (mouse)	Lin, Ogura, Margolskee, Finger, & Restrepo, 2008; Krasteva, Canning, Papadakis, & Kummer, 2012; Saunders, Reynolds, & Finger, 2013
Airway	Ciliated epithelial	Various T2R bitter	Increase ciliary beat	Unknown	Greene and Elvany,

(bronchi)	cells	receptors (human)	frequency and mucociliary clearance		2005; F. Li, 2013
	Smooth muscle	Various T2R bitter receptors (mouse and human)	Bronchodilation	Unknown	Saunders, Reynolds, & Finger, 2013; An et al., 2012; Deshpande et al., 2010; Robinett, Deshpande, Malone, & Liggett, 2011; pulkkinen et al., 2012
Auditory tube	Solitary chemosensory brush cells	T2R105, T2R108 bitter receptors (mouse)	Unknown; may release ACh and/or CGRP	Unknown	Grassin-Delyle et al., 2013
		T1R1/3 umami receptor (mouse)	Unknown; may release ACh and/or CGRP	Unknown	Grassin-Delyle et al., 2013
Bladder	Smooth muscle	T1R2/3 sweet receptor	Bladder contraction	Unknown	Krasteva, Hartmann, et al., 2012
Brain	Medulla oblongata	T2R1, 4, 107, 38 bitter receptors (rat)	Unknown	Unknown	Elliott et al., 2011; Dehkordi et al., 2012
	Hippo-campus, cornu ammonis fields, dentate gyrus	T2R2/3 sweet receptor (mouse)	Regulation of brain glucose homeostasis	CSF glucose concentration	Singh, Vrontakis, Parkinson, & Chelikani, 2011; Ren, Zhou, Terwilliger, Newton, &

					de Araujo, 2009
Breast	Mammary epithelial cells	T2R1,4,10,38, 49 bitter taste receptors (human)	Unknown	Unknown	Shin et al., 2010
Heart	Cardiac myocytes	Various T2R bitter receptors (mouse)	Unknown; upregulated by glucose starvation and proposed to be involved in "nutrient-sensing"		Singh, Chakrabort y, Bhullar, & Chelikani, 2014
Intestine	Enteroendoc rine/neuroen docrine cells	Various T2R bitter receptors (mouse and human)	Regulation of gastric emptying; influence on glucose homeostasis; contributing to avoidance responses of ingested toxic substances	Ingested "bitter" compounds (plant alkaloids, bacterial products, etc.)	Bezencon, le Coutre, & Damak, 2007; Dotson et al., 2008; hass et al., 2009; Foster et al., 2013
		T1R2/3 sweet receptor (mouse and human)	Secretion of glucagon-like peptide 1, glucose-dependent insulinotropic peptide, cholecystokin in; peptide YY; regulation of glucose transporter expression	Ingested sugars	Margolskee et al., 2007; Sclafani et al., 2007; Kokrashvili et al., 2009; Janssen et al., 2011; Shirazi-Beechey, Daly, Al-Rammahi, Moran, & Bravo, 2014

Pancreas	Beta-cells	T1R2/3 sweet receptor (mouse and human); potentially a T1R3 homo-oligomer	Potentiation of insulin secretion	Fructose	Schiffmann et al., 1999; Komji et al., 2014; Mayer-Gerspach et al., 2014
Testes	Seminiferous tubule cells; spermatids and spermatozoa	Various T2R bitter receptors (mouse and human)	Unknown	Unknown	Malaisse, Vanonderbergen, Louchami, Jijakli, & Malaisse-Lagae, 1998; J. Xu, Cao, Iguchi, Riethmacher, & Huang, 2013
	Spermatozoa	T1R1/3 umami receptors (mouse and human)	Regulation of sperm motility	Unknown	Li and Zhou 2012; Mayer et al., 2012
Urethra	Chemosensory brush cells	T2R bitter receptors	ACh release to stimulate bladder smooth muscle contraction	Uropathogenic *E. coli* cell components and/or secreted product(s)	Deckmann et al., 2014

Springer and Cellular and molecular Life Sciences, 72, 2015, 217-236, Taste receptors in innate immunity, Lee, R.J., Cohen N.A., table 1, © Springer Basel (outside the USA) 2014.

While expression of taste receptors in all these varied tissue types seems confusing, it is neither unusual nor novel. One good example is the expression of mGluRs as taste receptors. mGluRs are found in TSCs and there is strong evidence that suggests mGluRs are involved in umami taste. Long before mGluRs were identified in TSCs, they were well characterized and thoroughly studied in the CNS. In most cases these receptors

are activated by same ligand in different tissues, where the ligands direct tissue specific functions, thus regulating the receptor function.

1.6 Coming Back to the L-Amino Acid Story

1.6.1 Amino Acid Taste in Human:

The taste of amino acids in humans, in particular the taste qualities of D- and L-amino acids, has a long history of study. However, the early literature is controversial, in part because during the early 1900s, pure D- and L-amino acids were not readily available (Crocker, 1948; Berg, 1953; Ferguson & Lawrence, 1958; Lawrence & Ferguson, 1959; Stone, 1967). In general, while D-amino acids became associated mainly with sweet taste, L-amino acids were historically described as having a sweet or bitter taste (Solms, Vuataz, & Egli, 1965). Although L-glutamate was proposed to elicit a unique taste, umami, in the early 1900s (Ikeda, 2002), it was not until recently that western scientists began to accept umami as a basic taste. Umami, and to a lesser extent L-amino acid taste research, finally got a kick start following a major research symposium on umami in the mid-1980s.

Schiffman et al. (1981) analyzed the taste qualities and detection thresholds of the D- and L-amino acids by asking subjects to rank the taste of amino acids using a semantic differential scale (Schiffman, Sennewald, & Gagnon, 1981). However, the lack of any standard stimulus made it difficult to compare taste qualities between the amino acids. Later Birch and Kemp evaluated the taste of L-amino acids in relation to their size and water solubility (Birch & Kemp, 1989). While these studies provided information about the intensity of overall taste, they did not explain the complex taste elicited by L-amino

acids. Very recently the taste quality profiles of D- and L-amino acids were more precisely described. The taste of each amino acid was analyzed by focusing on the taste intensities of total taste experience and also individually for all five basic taste sensations (Kawai et al., 2012). This study reported that most of the L-amino acids elicit a complex taste in human, which is probably the result of activation of multiple receptors.

L-amino acids are vital components in our diets, and are available in many different foods in free form. In food preparation the contribution of amino acids, particularly the umami L-amino acids, often exceeds the taste properties of pure amino acids because of the phenomena of taste interaction, in this case specifically synergism. Here it is important to note that D-amino acids are also found in food. However, in most cases D-amino acids in dietary proteins originate as a consequence of various processing procedures used to cook or preserve food products for longer use or as a consequence of changes in the microbiological quality of the food (Friedman, 1999; Jin, Miyahara, Oe, & Toyo'oka, 1999; Albert & Csapo, 2009). The presence of these D-amino acids is often associated with a reduction in the digestibility of dietary protein and the availability of the transformed amino acid. Additionally, D-amino acids in food have been associated with various disease conditions, for example, renal problems. Thus, the presence of D-isomers of amino acids is mostly considered undesirable. Although some studies suggest that in certain cases D-amino acids can be beneficial to humans, more detailed research is needed. Since L-amino acids naturally occur in food and contain nutrition values, from now on we will focus our discussion mainly on L-amino acids.

Free L- amino acids are found in various protein rich foods such as meat, cheese, asparagus, etc. (Yamaguchi & Ninomiya, 1998, Yoshida 1998; Yamaguchi & Ninomiya, 2000). Furthermore, hydrolysis of proteins during aging, ripening, fermentation, and cooking liberates free glutamate and other L-amino acids (Yoshida, 1998). Digestion of protein, metabolic fate, and utilization of amino acids has been widely studied. Conversely, very little is known about the peripheral taste mechanisms for the detection of L-amino acids. Starting in the early 1990s, break-through progress was made in identifying taste receptors. Several umami receptors were identified by genetic analysis and molecular biology techniques. In 1990s a truncated variant of mGluR4 was proposed to detect L-glutamate in mice (Chaudhari et al., 1996, Chaudhari et al., 2000). In early 2000, the brain variant mGluR (Toyono et al., 2002) as well as a new receptor T1R1+T1R3 heterodimer was proposed to be involved in L-amino acid taste (G. Nelson et al., 2001, 2002; Li et al, 2002). Most physiological studies concerning the detection of L-amino acids are performed in mouse models, which in most cases, tally well with the physiological function of the proteins in human. Detection of L-glutamate by human taste receptors have been studied by in vitro approaches, using recombinant cellular models. While mouse T1r1+T1r3 expressed in HEK cell responded to many different L-amino acids, human T1R1+T1R3 responded mainly to L-glutamate+IMP mix (G. Nelson et al., 2002). Recently, the function of human sweet (T1R2+T1R3) and bitter (T2Rs) receptors are also studied using HEK cell expression system. This report showed that the D-isoform of amino acids preferentially bind to sweet receptors, which correlated well with the psychophysiological studies showing that most D-amino acids taste sweet. For T2Rs, amino acids activated them in an isoform dependent manner. In some cases, both

isoforms activated the same T2R (e.g., both D- and L-tryptophan activated T2R24 and T2R39), while in other cases, activation of a T2R was isoform specific (e.g., only L-tryptophan activated T2R43) (Bassoli, Borgonovo, Caremoli, & Mancuso, 2014). In the end the detection mechanisms of L-amino acids are still not understood.

1.6.2 In Search of L-amino Acid Receptor(s) – Rodent Story:

Several experimental approaches including behavioral, physiological, and molecular mechanisms have been undertaken to study the receptors for L-amino acids, although the focus has mainly been on the detection mechanisms for L-glutamate. In general 2 main types of behavioral taste tests are performed to study the response of rodents (rats and mice) to taste stimuli. These are: 1) Two-bottle preference test and 2) Discrimination test. Two-bottle preference test sessions generally are conducted for 24 or 48 hours to assess whether the animal has a positive or negative preference for the taste stimulus. In brief, animals are given free access to two bottles containing the same amount of liquid. One bottle contains the control solution, i.e, water (considered a neutral stimulus), and another bottle contains the test solution. At the end of the 24-hour test session, the volume consumed from each bottle is measured and the data are generally provided as a preference ratio of test solution to total volume consumed. If the ratio is 0.5, then the test solution is considered to have a neutral taste for which there is no preference. The test solution is considered preferable if the ratio is > 0.5, and aversive if the ratio is <0.5. However, one constrain of this test is any post ingestive effect of the taste stimulus, as this can influence the consumption of solutions. Therefore, it can be

difficult to have conclusive data when taste solutions are provided *ad libitum* over a long period of time, as it might be influenced by different physiological functions.

One effective alternative to 2-bottle preference is brief access testing. For this test animals are kept water deprived for a particular amount of time (12 to 24 hours) to motivate them to ingest solutions. Using an apparatus called a Davis Rig, animals are presented with test solutions in random order and the number of licks during test sessions are measured by a computer operated program. Since testing of each stimulus solution is usually very brief (e.g., 5-10 seconds) and the test session is often relatively short (e.g., less than 15 minutes), it is unlikely to be influenced by any post-ingestive effects. Typically during a 5-second trial, mice lick for water about 30-50 times. However the number is generally much lower for aversive and higher for preferable solutions.

Discrimination testing can be used as a more direct and robust assessment of taste. In brief, one test stimulus is repeatedly paired with a reinforcer (e.g., water or sucrose solution) to induce a specific response (e.g., increase lick rates) or a punisher (loud noise) to decrease lick rates. A second test solution is paired with the opposite consequence. Training will eventually lead to differential responding to each test solution. After animals are trained to discriminate between the tastes of the training solutions, additional test solutions may be presented to see if the rodent responds to them more like one or the other training solution, thereby indicating if the test solution elicits taste qualities similar to one of the training solutions. A variation of discrimination is conditional taste aversion (CTA) which is also popularly used to perform taste tests with rodents. In this method, the taste of a substance is paired with LiCl, which induces an upset stomach. The

aversion of the upset stomach is then associated with the taste solution. Thereafter, the mouse or rat (or human) will avoid the taste and, importantly, any stimulus which elicits a similar taste. The key feature of this test is that the greater the similarity in taste qualities between the two stimuli, the greater the avoidance of the second stimulus solution (stimulus generalization).

Earlier studies to identify L-glutamate taste were performed using MSG, the prototypical umami compound. In 1989, Ninomiya and Funakoshi showed that mice conditioned to avoid MSG do not generalize the aversion with any of the other basic taste or with other L-amino acids (Except some generalization with salt taste probably due to the sodium component of MSG). This suggested that MSG elicits a unique taste that does not generalize with other L-amino acids or with other basic tastes. However, the problem was the sodium component associated with MSG. When the taste of sodium was masked by an inhibitor for sodium channel, rodents were able to generalize the taste of glutamate with other L-amino acids, although the generalization was not always 100% (E. R. Delay, Sewczak, Stapleton, & Roper, 2004; E. R. Delay et al., 2007). This suggested that while L-amino acids elicit some taste very similar to L-glutamate, but the taste is not exactly the same as L-glutamate. In other words, L-amino acids elicit a complex taste. This further suggests L-amino acids also elicit a complex taste in human.

Molecular studies implicate the role of T1R1+T1R3 as an umami receptor (Li et al. 2002; G. Nelson et al., 2002). Very interestingly, while genetic knockout mouse models for this receptor validated the receptor function, it also brought controversy in the field of taste research. Zhao et al. (2003) showed that mice lacking T1r1 and T1r3

receptors completely lose their ability to taste umami or L-amino acids. However, in the same year, another group with independently generated knockout mice showed only reduced response in their T1r3 knockout mice (Damak et al., 2003). These differences can be attributed to how the knockout models were created. Nevertheless, other receptors were also identified in taste buds that can detect umami taste, which validated the findings by Damak et al. (2003). Glutamate receptor mGluR4 and mGluR1 are also expressed in taste epithelium, specifically in the epical end of the TSCs (Chaudhari et al 1996; Chaudhari et al., 2000; Toyono et al., 2002, 2003; San Gabriel et al., 2005; San Gabriel, Maekawa, Uneyama, & Tori, 2009). In addition to the brain expressed variants of these receptors, a truncated variant of the receptor is also found in TSCs. These truncated variants lack a large amount of N-terminal region and also possess a much lower affinity for L-glutamate compared to the brain variants (Toyono et al., 2002, 2003; San Gabriel et al., 2005; San Gabriel et al., Tori, 2009). Physiological and behavioral studies have provided evidence for the role of these receptors in umami taste. Bath application of a potent mGluR4 agonist L-AP4 has been shown to elicit responses in TSCs, which can also be suppressed by a potent mGluR4 antagonist *(RS)*-α-Cyclopropyl-4-phosphonophenylglycine (Lin & Kinnamon, 1999). Behavioral studies have reported that rodents perceive the L-AP4 taste as very similar to glutamate taste (Chaudhari et al., 1996; E.R Delay, Hernandez, Nakashima, Katsukawa, Sasamoto, & Ninomiya, 2001; Bromley & Margolskee, 2006). Additionally, in the presence of an mGluR4 antagonist, mice exhibited substantial reduction in CTA avoidance response to L-AP4 or glutamate. Recent molecular and behavioral studies using antagonists for mGluR1 have also

provided strong evidence for mGluR1 action in umami taste (Nakashima et al., 2001; Yasuo et al., 2001; Yoshida et al., 2009; Kusuhara et al., 2013).

1.6.3 Taste Potentiation:

Potentiation of glutamate response by IMP is a unique umami characteristic. Synergism between glutamate and 5'ribonucleotides was first found by Kuninaka (1960). Nerve recording studies have shown that responses to L-AP4 and MSG can be potentiated by IMP (Sako & Yamamoto, 1999). Additional behavioral and physiological studies have also shown the same result (E.R. Delay et al., 2000; Lin, Ogura, & Kinnamon, 2003). While the synergistic characteristic of these compounds is firmly established, the molecular mechanism is still unclear. Mutagenesis and receptor binding modeling studies have revealed the glutamate and IMP binding sites in the VFT domain of T1R1 subunit. Molecular modeling studies propose a unique mechanism for a positive allosteric modulatory function of IMP. According to the model, IMP exhibits synergy by binding to a site adjacent to the glutamate binding site, and further stabilizes the closed conformation of the VFT domain of the T1R1 subunit. Further, the model is confirmed by mutagenesis data (Zhang et al., 2008). However, these data do not explain individual IMP mediated responses.

1.7 Research Hypothesis

Even though studying the mechanisms for detecting umami taste has received enormous focus, detection and transduction mechanisms for L-amino acids are not thoroughly investigated. Similar to glutamate, other L-amino acids are found in food in free form. Both humans and mice can distinguish different L-amino acids (Kusahara et

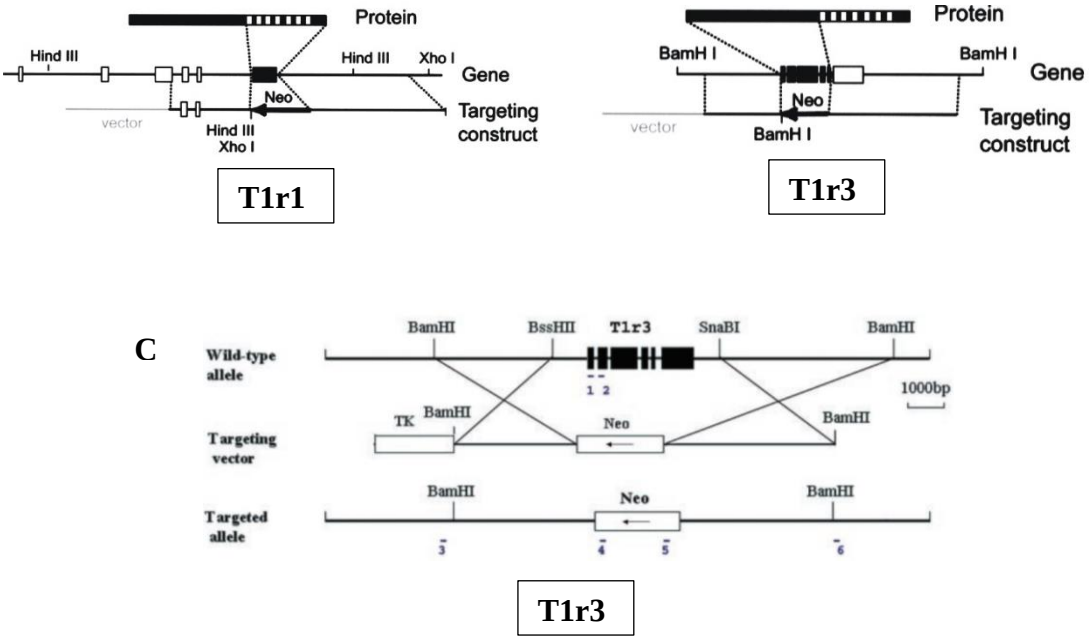
al., 1987; Kawai et al., 2012). In humans some L-amino acids elicits taste very similar to umami (L-aspertate), while other L-amino acids resemble completely distinct taste qualities (e.g., L-arginine, bitter). Making this even more complicated, some L-amino acids elicit a more complex taste in a concentration dependent manner. For example, at low concentration the L-amino acids serine and glutamine both taste sweet, but at high concentrations they elicit umami taste (Kawai et al., 2012). Similarly, rodents also generalize the taste of many L-amino acids with L-glutamate, however, these generalizations were not always completely bidirectional, suggesting a complex taste of L-amino acids (Kusahara et al., 1987; E. R. Delay et al., 2007).

Positive molecular identification of an L-amino acid receptor came from the heterogeneous expression of one of the candidate umami receptor, T1R1+T1R3. Mouse T1r1+ T1r3 responded to an array of L-amino acids, giving it the distinction of being a broadly tuned L-amino acid receptor. Additionally, for the L-amino acids, responses were potentiated in the presence of IMP (G. Nelson et al., 2002). However, the most interesting finding was that the receptor did not respond to glutamate presented alone, immediately indicating the involvement of another receptor. Similarly, the HEK cells expressing T1r1+T1r3 were also unable to generate any response to some other L-amino acids in absence of IMP, or to IMP alone, thus suggesting multiple receptors.

Contradictory to this, human T1R1+T1R3 when expressed in HEK cells, responded only to glutamate but not to other L-amino acids and the response was also potentiated by IMP (Li et al, 2002). Because L-amino acids elicit a complex taste (some bitter, some sweet, and some multiple taste modalities), the sweet and bitter receptors

might be the target receptors for these L-amino acids. On the other hand, there is no extensive study with all the L-amino acids and human T2Rs and T1R2+T1R3. There is evidence, however, that these receptors do not completely explain the taste mechanisms of some of the bitter and sweet tasting L-amino acids (Bassoliet al., 2014).

Further confirmation of the role of T1r1+T1r3 as umami and/or L-amino acid receptor came from studies with T1r1 and T1r3 knockout mouse. While this was a logical approach to study receptor function, research done by different labs using independently developed mice models reported very different results, adding further confusion (Damak et al., 2003; Zhao et al., 2003; Kusuhara et al., 2013). While this sounds perplexing, the differences in these outcomes are likely attributed to how the knockout models were engineered.



(A, B) Adapted by permission from Macmillan Publishers Ltd: [NATURE] (Chandrashekar, J., Hoon, M. A., Ryba, N. J., & Zuker, C. S. The receptors and cells for mammalian taste. 444(7117), 288-294. doi: 10.1038/nature05401) copyright (2006)

(C) Adapted from Damak, S., Rong, M., Yasumatsu, K., Kokrashvili, Z., Varadarajan, V., Zou, S., . . . Margolskee, R. F. (2003). Detection of sweet and umami taste in the absence of taste receptor T1r3. *Science, 301*(5634), 850-853. doi: 10.1126/science.1087155

Figure 1.7: Schematic diagram showing how knockouts are created by different labs.
(A) Targeting vectors were designed to eliminate the exons for the transmembrane domain, (B) Targeting vectors were designed to eliminate the exons for the N-terminus domain, (C) Targeting vectors were designed to eliminate all the T1r3 coding exons. A and B are Adapted from Damak et al., 2003. C was adapted from Zhao et al., 2003.

In mice created by Zhao et al., (2003), only exons for the N-terminal region were deleted in T1r3 KO mice whereas the transmembrane region was eliminated in the T1r1 KO mice. In contrast, all the exons were deleted in the T1r3 KO mice created by Damak et al. (2003), or the T1r1 KO mice created by Kusahara et al. (2012). Although the knockout approach used by Zhao et al. should, in theory, eliminate the functional expression of the receptor, it is possible that expression of a partial receptor blocks the function of other receptors in a dominant negative mechanism and thereby also hampers proper functioning of other receptors. Nevertheless there are several studies that provide strong evidance for multiple receptors in umami taste (E. R. Delay et al., 2006; Maruyama et al., 2006). Additional candidate receptors for umami taste include brain as well as taste specific varients of mGluR1 and mGuR4. The taste-mGluR4 was found to be missing more than 50% of the N-terminal region compared to the brain-variant of mGluR4 (Chaudhari et al., 2000). Like the better known brain-mGluR4, the truncated taste variant responds to glutamate and the group III selective mGluR agonist L-(+)-2-amino-4-phosphonobutyrate (L-AP4) (Chaudhari et al., 1996; Hayashi et al., 1996; Bigiani, Delay, Chaudhari, Kinnamon, & Roper, 1997), although the affinity of taste-mGluR4 for glutamate and L-AP4 is more than 100 times lower than that of brain-type receptors (Chaudhari et al., 1996, Chaudhari et al., 2000). Similarly a full length variant of mGluR1 was identifies in TSCs (Toyono et al., 2003) as well as a truncated variant of

mGluR1 (taste-mGluR1) (San Gabriel et al., 2005; San Gabriel et al., 2009; Nakashima, Eddy, Katsukawa, Delay, & Ninomiya, 2012). Like taste-mGluR4, the truncated taste-mGluR1 also lacks much of the N-terminal extracellular domain and has more than 100-fold lower affinity for glutamate than does the brain-variant of the receptor (San Gabriel et al., 2005; San Gabriel et al., 2009). The full-length brain-mGluR1 and brain-mGluR4 are now known to be expressed in a subset of taste cells in fungiform, foliate and circumvallate papillae in rats in contrast to the truncated taste variants of mGluR1 and mGluR4 which are mainly expressed in the foliate and circumvallate papillae. Behavioral, nerve recording, and calcium imaging studies with rodents have shown that antagonists for mGluR4 and mGluR1 can selectively block or reduce responses elicited by glutamate (Nakashima et al., 2001; Eschle, Eddy, & Delay, 2009; Yoshida et al., 2009; Kusuhara et al., 2013).

While much is known about glutamate transduction, detection mechanisms of other L-amino acids are less well understood. Understanding the receptor system and transduction mechanisms for L-amino acids is noteworthy because L-amino acids function as the building blocks of proteins and as metabolic fuel. Having more than one receptor for detecting these compounds would be advantageous. One such candidate receptor is the T1r1+T1r3 as it is reported to be a broadly tuned L-amino acid receptor (G. Nelson et al., 2002). Interestingly, IMP potentiates the response for several L-amino acids in HEK cells (G. Nelson et al., 2002). Like umami, this property makes understanding L-amino acid detection mechanisms particularly important as they could be targets for altering taste properties of food, making it more or less desirable.

My approach in advancing the understanding of L-amino acid taste perception was to use a mouse model to study the receptor system for L-amino acid detection. My general hypothesis was: **Umami transduction is elicited by L-glutamate and other L-amino acids, and the transduction mechanisms of L-amino acids are the same as L-glutamate.**

If a single receptor is involved in the detection of all L-amino acids, then all L-amino acids should have the same or very similar taste properties. However, not all L amino acids elicit the same taste. Some L-amino acids are attractive to rodents, some are aversive. Human psychophysical studies have showed that at low concentrations, L-serine (Ser) and L-glutamine (Gln) elicit a sensation that is mainly sweet, whereas L-arginine (Arg) is bitter. Further, at high concentrations, Ser and Gln elicit an umami taste (Kawai et al., 2012). Additional studies with rats have shown that rats are differentially sensitive to MSG, Ser, and Arg (E. R. Delay et al., 2007). Collectively, these data suggest the possibility of multiple L-amino acid receptors.

Here I first investigated TSCs from mice circumvallate and foliate papillae located at the posterior portion of the tongue, to explore the nature of the responses and potential receptors involved in the detection of L-amino acids. I used calcium (Ca^{2+}) imaging of isolated TSCs and taste cell clusters to determine if: 1) single TSCs are responsive to a set of four L-amino acids, with and without IMP, and to IMP alone, 2) TSCs respond synergistically to the MIX of L-amino acid+IMP, and 3) TSCs of T1r3 KO mice can detect L-amino acids and respond synergistically in the presence of IMP. I found that the response patterns elicited by L-amino acids varied significantly across

TSCs. L-amino acids other than glutamate also elicited synergistic responses in a subset of TSCs. Along with its role in synergism, IMP itself also elicited a response in TSCs. My study suggests that in addition to the T1r1+T1r3 heterodimer, another receptor or possibly receptor complex is/are involved in the detection of L-amino acids and IMP.

The next step of the study was to identify which other receptors detect L-amino acids in TSC. Since mGluRs have already been established to function as umami receptors, I first tested if mGluRs might also be involved in L-amino acids and IMP detection. To address this question, I recorded L-amino acid and IMP-elicited calcium responses of TSCs from T1r3-GFP labeled mice and T1r3 KO mice to identify the involvement of different mGluRs in the detection of various L-amino acids and IMP. The three L- amino acids used in this study are: (1) monopotassium glutamate (MPG), (2) Ser, and (3) Arg - all of which are known to elicit different tastes in mice and humans, although CTA experiments with rats suggest these amino acids may also share some taste qualities (Schiffman et al., 1981; E.R. Delay et al., 2007; Eschle et al., 2009; Kawai et al., 2012). I specifically studied the involvement of mGluR1 and mGluR4 in L-amino acid and IMP detection by examining the effects of selective mGluR1 and mGluR4 agonists on TSCs and the effect of selective antagonists on responses elicited by L-amino acids and IMP. I found that some TSCs respond to selective agonists also responded to L-amino acids and IMP. Additionally, selective antagonists for both mGluR1 and mGluR4 significantly suppressed the responses elicited by L-amino acids and IMP. The results suggest the involvement of both brain and taste variants of mGluR1 and mGluR4 in detection of IMP and L-amino acids. In summary, the results presented in this

dissertation provide strong evidence for a multiple receptor system for L-amino acids and IMP detection.

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Chapter 2 : L-AMINO ACIDS ELICIT DIVERSE RESPONSE PATTERNS IN TASTE SENSORY CELLS: A ROLE FOR MULTIPLE RECEPTORS

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2.1 Abstract

Umami, the fifth basic taste, is elicited by the L-amino acid, glutamate. A unique characteristic of umami taste is the response potentiation by 5' ribonucleotide monophosphates, which are also capable of eliciting an umami taste. Initial reports using human embryonic kidney (HEK) cells suggested that there is one broadly tuned receptor heterodimer, T1r1+T1r3, which detects L-glutamate and all other L-amino acids. However, there is growing evidence that multiple receptors detect glutamate in the oral cavity. While much is understood about glutamate transduction, the mechanisms for detecting the tastes of other L-amino acids are less well understood. We used calcium imaging of isolated taste sensory cells and taste cell clusters from the circumvallate and foliate papillae of C57BL/6J and T1r3 knockout mice to determine if other receptors might also be involved in detection of L-amino acids. Ratiometric imaging with Fura-2 was used to study calcium responses to monopotassium L-glutamate, L-serine, L-arginine, and L-glutamine, with and without inosine 5' monophosphate (IMP). The results of these experiments showed that the response patterns elicited by L-amino acids varied significantly across taste sensory cells. L-amino acids other than glutamate also elicited synergistic responses in a subset of taste sensory cells. Along with its role in synergism, IMP alone elicited a response in a large number of taste sensory cells. Our data indicate that synergistic and non-synergistic responses to L-amino acids and IMP are mediated by multiple receptors or possibly a receptor complex.

2.2 Introduction

The sense of taste provides vital sensory information to determine whether a particular food or beverage will be ingested. It is integral for regulating normal ingestive decisions and is particularly important to people experiencing any disease conditions such as obesity, diabetes, hypertension, coronary artery disease, anorexia, and malnutrition [1-11]. Detection of taste stimuli is mediated by the coordinated actions of distinct types of taste sensory cells (TSCs) housed in taste buds of specialized papillae in the oral cavity. Taste receptors in TSCs that detect compounds eliciting sweet, salty, sour, bitter, and umami tastes are the key players in selecting nutrients. One such example is amino acids that are an important part of one's diet.

Each basic taste quality generally signals a fundamental type of nutrient. For example, sweet taste is often considered a general signal for carbohydrates in food whereas umami taste is thought to signal the presence of proteins and nucleotides. Umami taste is characterized by two distinctive qualities: 1) a unique savory taste, and 2) synergism with 5' nucleotide monophosphates, especially inosine 5' monophosphate (IMP) and guanosine 5' monophosphate (GMP) [12, 13]. The prototypical compound that elicits umami taste in humans is monosodium glutamate (MSG), a substance known to increase the palatability of food [14-16]. Recent research has shown that fortification of meals with an appropriate amount of MSG may improve food intake and therefore has potential for improving nutritional status and quality of life in elderly and nutritionally deficient patients [16-19]. Thus, understanding the receptors and transduction pathways

that mediate umami taste could be beneficial in regulating the intake of nutrients that are critical for clinical populations with dietary challenges.

Umami compounds are detected by receptors expressed in Type II TSCs [20-25]. A long standing question concerning umami taste relates to whether umami and L-amino acids are detected by one receptor or multiple receptors. Previous studies including *in vitro* receptor expression, behavioral, nerve recording, and single cell recording experiments have suggested that members of the T1r receptor family form a heterodimer, T1r1+T1r3, which is an umami receptor in mice [23, 24]. Further support for its role as an umami receptor comes from studies with knockout (KO) mice in which *Tas1r1* or *Tas1r3* gene was selectively eliminated. Some of these studies have shown that these mice lose all ability to respond to umami stimuli [25]. However, other studies with independently derived T1r1 and T1r3 receptor KO mice found only partial taste loss for umami [22, 26, 27]. Additional studies have reported that other G-protein coupled receptors (GPCRs) such as truncated variants of mGluR4 (taste-mGluR4) and mGluR1 (taste-mGluR1), as well as the brain versions of mGluR4 and 1 may be involved in the detection of umami compounds [20, 21, 28–30]. Moreover, there is evidence for expression of mGluR2 and mGluR3 in taste buds [31]. Together these studies argue for the involvement of more than one receptor that can detect umami compounds.

While much is known about glutamate transduction, detection mechanisms of other L-amino acids are less well understood. Understanding the receptor system and transduction mechanisms for L-amino acids is noteworthy because L-amino acids function as the building blocks of proteins and as metabolic fuel. Having more than one

receptor for detecting these compounds would be advantageous. One such candidate receptor is the T1r1+T1r3 heterodimer. Transfected human embryonic kidney (HEK) cell expression data suggest that the murine heterodimer T1r1+T1r3 is a broadly tuned L-amino acid receptor [24]. Behavioral data suggest that one or more mGluR receptors may also detect some amino acids [32, 33]. Although IMP potentiates the response for several L-amino acids in HEK cells, some L-amino acids could elicit a response only in the presence of IMP [24]. Like umami, this property makes understanding L-amino acid detection mechanisms particularly important as they could be targets for altering taste properties of food, making it more or less desirable.

If a single receptor is involved in the detection of all L-amino acids, then all L-amino acids should have the same or very similar taste properties. However, not all L amino acids elicit the same taste. Some L-amino acids are attractive to rodents, some are aversive. Human psychophysical studies have showed that at low concentrations, L-serine (Ser) and L-glutamine (Gln) elicit a sensation that is mainly sweet, whereas L-arginine (Arg) is bitter. Further, at high concentrations, Ser and Gln elicit an umami taste [34, 35]. Additional studies with rats have shown that rats are differentially sensitive to MSG, Ser, and Arg [33]. Collectively, these data suggest the possibility of multiple L-amino acid receptors.

In this study, we investigated TSCs from mice circumvallate and foliate papillae located at the posterior portion of the tongue, to explore the nature of the responses and potential receptors involved in detection of L-amino acids. We focused on the posterior portion of the tongue for several reasons. First, the posterior portion of the tongue has

been shown to generate a strong response to umami and L-amino acid stimuli [36]. Second, the circumvallate and foliate papillae are much richer in TSCs compared to the fungiform papillae. Third, the pattern of expression of different receptors varies between the posterior and anterior portion of the tongue. Since this might contribute to differences in response patterns, we chose to study TSCs in the posterior part of the tongue to reduce potential sources of variability and enhance our ability to identify response patterns across TSCs. We used calcium (Ca^{2+}) imaging of isolated TSCs and taste cell clusters to determine if: 1) single TSCs are responsive to a set of four L-amino acids, with and without IMP, and to IMP alone, 2) TSCs respond synergistically to the MIX of L-amino acid+IMP, and 3) TSCs of T1r3 KO mice can detect L-amino acids and respond synergistically in presence of IMP. We found that the response patterns elicited by L-amino acids varied significantly across TSCs. L-amino acids other than glutamate also elicited synergistic responses in a subset of TSCs. Along with its role in synergism, IMP itself also elicited a response in TSCs. Our study suggests that in addition to the T1r1+T1r3 heterodimer, another receptor or possibly receptor complex is/are involved in the detection of L-amino acids and IMP.

2.3 Materials and Methods

2.3.1 Ethical Consideration:

All experimental procedures were reviewed and approved by the University of Vermont's Institutional Animal Care and Use Committee (IACUC protocol: 10-038). Mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. All efforts were made to minimize suffering.

2.3.2 Animals:

Male and female (>8 weeks old) C57BL/6J (WT) (Jackson labs), T1r3-GFP [37], and T1r3 KO [22] mice were used in this study. T1r3-GFP mice express enhanced green fluorescent protein (eGFP) under control of the *Tas1r3* gene promoter and were generated on C57BL/6J background. The T1r3-GFP mice were primarily used in the early phases of the study to help identify isolated TSCs. T1r3KO mice were generated on C57BL/6J background, and all 6 exons for the *Tas1r3* gene were eliminated [22]. Breeding stock for T1r3-GFP and T1r3 KO mice were generously donated by Dr. Robert Margolskee [22, 37]. GFP expression and genetic deletion of *Tas1r3* gene were verified by polymerase chain reaction (PCR). For clarity, throughout the paper we are using T1r1 and T1r3 to refer to the receptor proteins in mice. All mice were maintained on a 12-h light/12-h dark cycle with food and water provided *ad libitum*.

2.3.3 Solutions:

Tyrode's solution contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, Glucose 10, and Na pyruvate 1. High potassium (high K^+) Tyrode's solution contained the same constituents as regular Tyrode's solution with the exception that 65 mM KCl was substituted for equimolar NaCl. Ca^{2+}/ Mg^{2+} free Tyrode's contained (in mM): NaCl 140, KCl 5, HEPES 10, Glucose 10, Na pyruvate 1, and EGTA 2. L-amino acids used as test solutions were (in mM): L-Arg 10, L-Ser 20, L-Gln 10, and monopotassium L-glutamate (MPG) 10. MPG was used to ensure that responses were not due to the sodium component of MSG. In many studies MPG has been successfully used to reliably evoke taste responses to the glutamate moiety [38, 39]. Additionally, 10mM of

K^+ is not sufficient to cause the amount of depolarize required to activate L-type Ca^{2+} channels expressed in TSCs. Di-sodium inosine 5' monophosphate (IMP) was used at 1mM. The addition of 2mM sodium associated with IMP was very small compared to the amount of Na^+ (140mM) in the bath and thus unlikely to elicit any cellular responses. Physiologically relevant stimulus concentrations for each substance were chosen from behavioral and physiological data to ensure that each concentration was above recognition threshold in rodents (mice and rats) [28, 36, 40, 41], but not high enough to cause any osmotic changes. The artificial sweetener, SC45647 (2-[[[[4-(aminomethyl)phenyl]amino]-[[(1*R*)-1-phenylethyl]amino]methyl]amino]ethane-1,1-diol) (100μM) was used as a sweet stimulus [42], and denatonium (2mM) or a mixture of cycloheximide (20μM) and denatonium (2mM) was used as a bitter stimulus. The L-amino acids, sweet, and bitter compounds were dissolved in Tyrode's solution and made fresh every day. When generating a mixture solution (MIX) of L-amino acid+IMP, the concentration of each compound was the same as those used for the individual compounds. In the MIX, 1mM IMP was mixed with 10mM of either of MPG, Arg, Gln, or 20mM of Ser. In the AA-MIX (L-amino acid-MIX), all four L-amino acids (MPG, Ser, Arg, and Gln) were used at the same concentration as mentioned before. All solutions were adjusted to approximately pH 7.4 using NaOH or HCl.

2.3.4 Taste cell isolation:

Taste cells from circumvallate and foliate taste buds were isolated using a protocol adapted from Behe et al. [43] and Gilbertson et al. [44]. In short, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. Tongues were removed

and immersed in ice cold Tyrode's solution. The lingual epithelium was removed by injecting an enzyme cocktail containing 0.8 mg/mL collagenase A (Sigma, St. Louis, MO), 1.5 mg/mL dispase II (Roche, Indianapolis, IN), 1 mg/mL trypsin inhibitor (Sigma, St. Louis, MO), and 0.05mg/mL elastase (Worthington, Lakewood, NJ) directly under the epithelium. The tongue was then incubated in Tyrode's solution for 20 min followed by incubation in Ca^{2+}/ Mg^{2+}-free Tyrode's for another 20 min. In both solutions, oxygen was supplied continuously. The epithelium was gently removed from the underlying connective tissue and pinned flat with the epithelium surface down on a sylgard-lined petri dish. The tissue was incubated in the enzyme cocktail (without dispase II) for 5 min before being transferred to Ca^{2+}/Mg^{2+} free Tyrode's solution for 20-25 min. Taste buds and TSCs were removed from circumvallate and foliate papillae by gentle suction using fire polished glass micropipettes. TSCs were plated into a shallow recording chamber with a glass cover-slip pre-coated with Concanavalin A (Sigma, St. Louis, MO) to promote cell adherence. This protocol enabled us to reliably obtain both isolated taste cells and clusters of taste cells. Typically the cells were viable for 6-7 hours.

2.3.5 Calcium (Ca^{2+}) imaging:

Measurements of intracellular Ca^{2+} were obtained using the ratiometric fluorescent dye fura-2 AM (Molecular probes, Invitrogen Corporation, NY). Taste cells were incubated in 5-μM fura-2, AM and 0.05% pluronic F-127 dissolved in DMSO in Tyrode's solution for 25-30min. The recording session began after bath washing the cells in Tyrode's solution for 10-20min. Images were acquired using an inverted fluorescent Nikon TE2000S microscope and C4742-95 digital camera. All solutions were bath

applied using a gravity flow perfusion system. Stimuli were applied for 30s before returning to Tyrode's solution. Application of stimuli was in random order to avoid any systemic error. Sometimes a MIX of L-amino acid+IMP was applied after the L-amino acid, and sometimes the MIX was applied before the L-amino acid. In both instances we found some cells that elicited synergistic responses. Thus synergistic or non-synergistic responses were not dependent on the stimulus application sequence. At the end of any stimulus application, cells were washed in Tyrode's solution for 5 to 9 min. We performed a desensitization study in which single cells were stimulated with the same stimulus 4 to 5 times with varying wash times in between. We found that a wash of 5 to 9 min between stimulus applications was optimal for repeated responses of similar magnitude, although in some cells desensitization occurred independent of an extended wash. If the final stimulus application in the test sequence did not elicit a response, we stimulated the cell with a compound which previously elicited a response to make sure that the cell was still alive. Images were captured every 3 s during stimulus application and every 5 to 15 s during wash. In order to minimize cell damage during long wash periods, we limited image capturing during washes. After a response, images were captured only until the Ca^{2+} level went back to baseline (~2-3 min after the start of stimulus application) and image capturing was resumed at least 1 min prior to next stimulus application. Fura2 AM was doubly excited at 340nm and 380nm and its emissions were recorded at 510nm. Simple PCI 6.0 software (Hamamatsu, Sewickley, PA) running on a PC computer was used to capture images. Changes in Ca^{2+} concentrations are reported as F340/F380 plotted over time after background subtraction.

2.3.6 Quantification of calcium responses:

Increases in intracellular Ca^{2+}evoked by stimulus application were calculated as follows:

$$\Delta F = \frac{F_{Peak} - F_{Baseline}}{F_{Baseline}} * 100\%$$

where ΔF is the percent change above baseline, F_{Peak} is the largest F340/380 ratio within 1 min following the onset of a stimulus application, and $F_{Baseline}$ is the average of the five sampled F340/380 ratios immediately before stimulus application. Our criteria for considering ΔF a response were: 1) the same stimulus elicited an increase in intracellular Ca^{2+} at least twice, and 2) each ΔF≥5%. Furthermore, ΔF was considered a synergistic response when:

$$\Delta F_{MIX} > \Delta F_{AA} + \Delta F_{IMP}$$

where ΔF_{MIX} is the percent change above baseline following application of a MIX of L-amino acid (where L-amino acid can be MPG, Ser, Arg, or Gln)+IMP, ΔF_{AA} is the percent change above baseline following application of MPG, Ser, Arg, or Gln alone, and ΔF_{IMP} is the percent change above baseline following application of IMP alone. Although peak Ca^{2+} is typically used to identify synergistic responses, we recognized that synergy might appear as an increase in duration of the signal instead of an increase in peak amplitude of the signal. To determine if we missed a large subset of potential synergistic responses, we measured the area under the curve of the responses classified as non-synergistic by peak amplitude. Only 2 cells from the Arg set and 3 cells from the Gln set were identified as potentially synergistic using the integrated response. Since these

responses might also represent continued stimulation by residual stimulus solution rather than synergy, they were excluded from the analyses involving synergy in favor of the more reliable measure of peak amplitude.

2.4 Results

2.4.1 Single TSCs from WT Mice Respond to Multiple, but Not All L-Amino Acids:

To better understand how TSCs respond to L-amino acids, and to determine if the T1r1+T1r3 heterodimer is the only receptor involved in L-amino acid detection, we investigated whether a single TSC would show a Ca^{2+} response to an array of L-amino acids with and without IMP. We isolated single TSCs and taste cell clusters from mouse circumvallate and foliate papilla. Each TSC was stimulated with 9 different stimuli: (1) MPG, (2) Ser, (3) Arg, (4) Gln, (5) IMP, (6) MPG+IMP, (7) Ser+IMP, (8) Arg+IMP, and (9) Gln+IMP. All test solutions were applied regardless of whether the cell responded to any individual stimulus. A total of 600 out of 1217 (49%) TSCs were successfully tested with all 9 stimuli, where the cells were alive until the end of the experiment. At least one stimulus was capable of eliciting a Ca^{2+} response (i.e. $\Delta F \geq 5\%$ in response to stimulation) in 170 of 600 (28%) TSCs. Thus we focused our analysis on the 170 cells that had a Ca^{2+} response to at least one stimulus.

Like previous studies [25, 36, 45], we found that TSCs responded to L-amino acids when presented with IMP. Out of 170 responsive TSCs, 133 (78%) responded to MPG+IMP, 118 (69%) responded to Ser+IMP, 111(65%) responded to Arg+IMP, and

114 (67%) responded to Gln+IMP. TSCs also responded to the L-amino acids MPG, Ser, Arg, and Gln when presented individually (Table 2.1).

Table2.1: Summary of responsive WT and T1r3 KO TSCs to the 9 stimuli.

	WT		**T1r3 KO**	
	Total number of cells successfully tested with all 9 stimuli: 600		**Total number of cells successfully tested with all 9 stimuli: 154**	
	Number of cells with response to any stimulus: 170 (28%)		**Number of cells with response to any stimulus: 24 (16%)**	
Stimulus	**No. of Responsive cells out of 170 cells (%)**	**No. of Synergistic Cells**	**No. of Responsive cells out of 24 cells**	**No. of Synergistic Cells**
IMP	121 (71)	N/A	14 (58)	N/A
MPG	78 (46)	N/A	16 (67)	N/A
MPG+IMP	133 (78)	87	21 (87)	9
Ser	65 (38)	N/A	24 (100)	N/A
Ser+IMP	118 (69)	59	24 (100)	15
Arg	63 (37)	N/A	18 (75)	N/A
Arg+IMP	111 (65)	70	23 (96)	14
Gln	66 (39)	N/A	18 (75)	N/A
Gln+IMP	114 (67)	75	16 (67)	2

Values are number of cells. Values in parenthesis are percentages.

In previous research, HEK cells transiently transfected with the T1r1+T1r3 heterodimer, exhibited Ca^{2+} responses to the umami compound L-glutamate, but only when IMP was present. Notably, IMP alone had no effect on HEK cells [24]. In contrast, we found that MPG presented alone elicited Ca^{2+} responses in 78 of 170 (46%)

responsive TSCs. The mean amplitude of MPG (10mM) evoked Ca^{2+} response (ΔF) was 32.38±5.3% (Mean±SEM) above baseline (Table 2.1; Fig. 2.1A Cell 1 and Cell 3, 1B). In addition, IMP presented alone elicited a Ca^{2+} response of 19.20±1.48% (Mean±SEM) above baseline in 121 of 170 (71.17%) responsive TSCs (Table 2.1; Fig. 2.1A Cell 3, 2.1B).

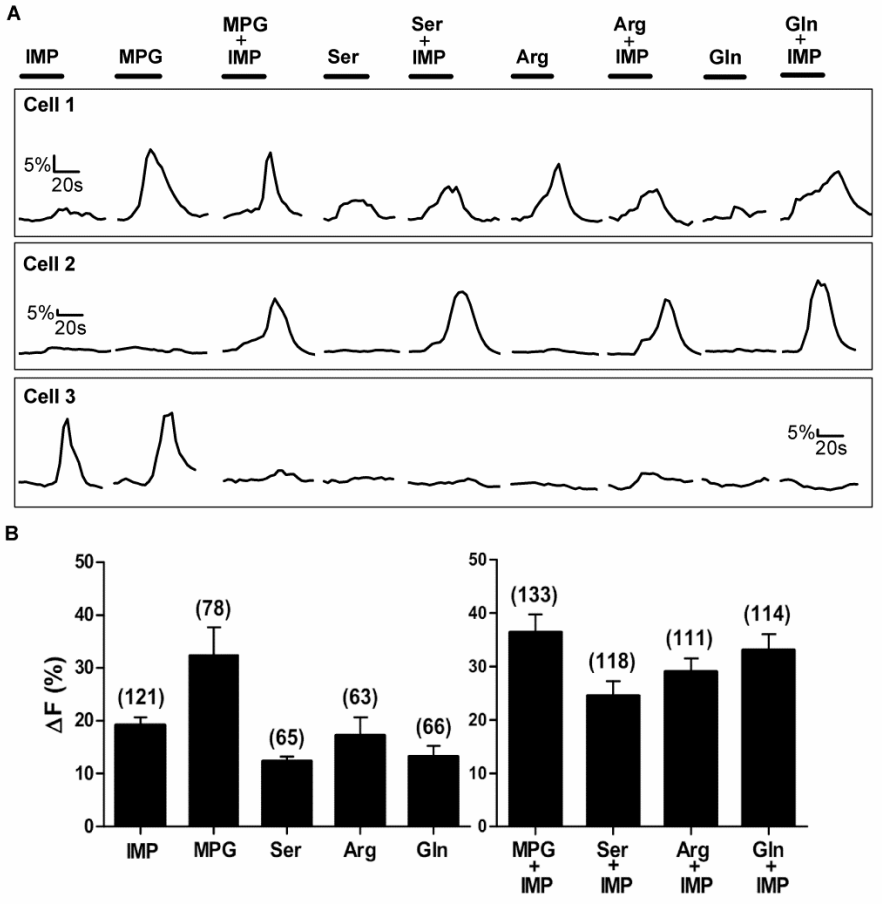

Figure 2.1: Representative Ca^{2+} responses of 3 TSCs from WT mice.
Stimuli tested were IMP (1mM), 4 L-amino acids from different side-chain groups (MPG (10mM), Ser (20mM), Arg (10mM), and Gln (10mM)), and L-amino acids with IMP. [A] Ca^{2+} responses of 3 sample TSCs. Each cell was tested with all 9 stimuli. The responses of the three cells are examples of some of the different response patterns to the array of stimuli. The bar above each stimulus trace represents stimulus application time (30 sec). [B] Mean±SEM amplitude of Ca^{2+} increase above baseline for responsive cells only, i.e. cells with a change in Ca^{2+} ≥5%. Numbers in parenthesis are the number of cells.

In our study, a single TSC when stimulated with 9 different stimuli, often responded to more than one L-amino acid but did not necessarily respond to all of the test solutions. For example, cell 1 in Fig. 2.1A, responded to all 9 stimuli tested, whereas cell 2 in Fig. 2.1A responded to IMP and to the L-amino acids only when presented with IMP. Clearly, cell 2's responses to each of the L-amino acid+IMP-MIXes were not elicited solely by IMP, as response magnitudes to the MIXes (35-45% above baseline) were much greater than the response magnitude to IMP (5% above baseline) alone.

Only 10 of 170 (6%) TSCs, responded to all 9 stimuli, while the remaining TSCs responded to some but not all stimuli. Moreover, TSCs did not always respond to L-amino acids when presented with IMP (Table 2.1; Fig. 2.1). Analyzing TSCs with responses to only L-amino acids, irrespective of their responses to IMP or any MIX of L-amino acid with IMP, we found 21 of 170 (12%) TSCs responded only to MPG but not to any of the other three L-amino acids tested (Ser, Arg, and Gln). Another 57 of 170 (33%) TSCs responded to MPG and to one or more of the other three L-amino acids tested. On the other hand, 54 of 170 (31%) TSCs did not respond to MPG but did respond to one or more of the other three L-amino acids. These results suggest that all L-amino acid responsive cells do not necessarily respond to the prototypical umami L-amino acid, glutamate. In addition, more than three-fourths of the TSCs responded to 1mM IMP, suggesting that IMP may be detected by a mechanism that is independent of the T1r1+T1r3 heterodimer.

2.4.2 In some but not all cases, L-amino acids elicit synergy when presented with IMP:

Synergy between 5' ribonucleotides (IMP and GMP) and L-glutamate is a defining characteristic of umami taste. The basis for this effect begins in the TSC and the taste bud. Previous studies [25, 36, 45] have shown that MPG elicited synergistic responses in TSCs when mixed with IMP or GMP. In addition to increasing response intensity, GMP also increased the number of responsive TSCs [45]. Similar to previous studies, we found a greater number of TSCs responsive to the MIX of MPG+IMP. For example, 70% more TSCs responded to MPG in presence of IMP than to MPG alone. In the same way, IMP also increased the number of cells responding to other L-amino acids. For instance, 81%, 76%, and 72% more cells responded to Ser, Arg, and Gln, respectively, in the presence of IMP compared to the L-amino acid alone (Table 2.1). We next analyzed the peak amplitude of MIX (L-amino acid+IMP) responsive TSCs to determine which Ca^{2+} responses were synergistic and if L-amino acids other than glutamate also elicited synergistic responses. Of the 170 TSCs, 133 (78%) TSCs responded to the MPG+IMP-MIX, 118 (69%) TSCs responded to the Ser+IMP-MIX, 111(65%) TSCs responded to the Arg+IMP-MIX, and 114 (67%) TSCs responded to the Gln+IMP-MIX.

For each of the L-amino acids tested, only a subset of MIX-responsive cells elicited synergistic responses (Table 2.1; Fig. 2.2). Approximately 50-65% of the MIX-responsive TSCs responded synergistically to one or more of the amino acids. Of the 133 MPG+IMP-MIX-responsive TSCs, 87 (65%) cells showed synergistic responses. Clearly,

not all MPG+IMP-MIX-responsive cells were synergistic (Table 2.1). For synergistic responses, the average increase in intracellular Ca^{2+} response to MIX was significantly greater than the sum of the responses to the individual stimulus compounds (One way ANOVA; $P<0.0001$). In addition, the mean Ca^{2+} increase for synergistic responses of these cells was significantly greater than the MIX response of non-synergistic cells (One way ANOVA; $P<0.0001$) (Fig. 2.2). We similarly analyzed the Ca^{2+} responses to the MIXes of Ser+IMP, Arg+IMP, and Gln+IMP. Of the 118 Ser+IMP-MIX-responsive cells, 59 (50%) cells exhibited synergistic responses. Likewise, 70 of 111 (63%) Arg+IMP-MIX-responsive cells, and 75 of 114 (65%) Gln+IMP-MIX-responsive cells showed synergistic responses (Table 2.1). Like the MPG+IMP-MIX-responsive TSCs, the magnitudes of the Ca^{2+} responses of TSCs that responded synergistically to the MIX for each of the L-amino acids were significantly greater than MIX responses of non-synergistic cells (Fig. 2.2).

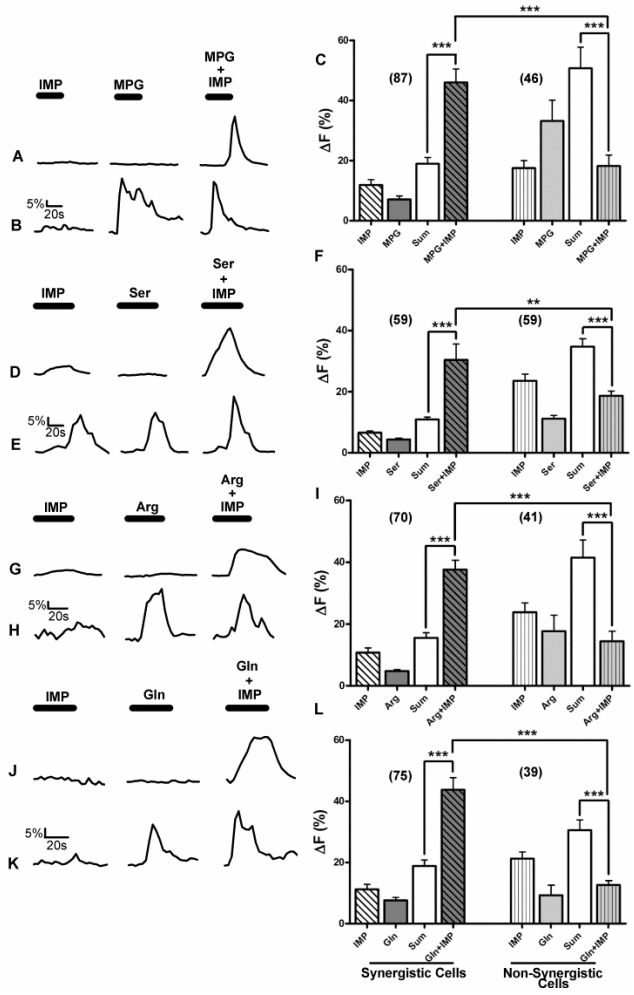

Figure 2.2: Some but not all TSCs generated synergistic response to the L-amino acid+IMP MIXes. Ca^{2+} responses of WT TSCs to the four L-amino acid sets are shown. Each L-amino acid set consisted of one of the four different L-amino acids (MPG (10mM), Ser (20mM), Arg (10mM), or Gln (10mM), respectively), IMP (1mM), and the MIX of L-amino acid+IMP. [A, D, G, J] Representative TSC responses where the magnitudes of MIX responses were greater than the summation of individual L-amino acid and IMP responses, i.e., the MIX responses were synergistic. [B, E, H, I] Representative TSC responses where the magnitudes of MIX responses were greater than or equal to the responses of the L-amino acid or IMP individually, but not greater than the summation of individual L-amino acid and IMP responses, i.e., the MIX responses were not synergistic. [C, F, I, L] A Mean±SEM response for the L-amino acid sets exhibiting synergistic and non-synergistic responses. For each L-amino acid set, the MIX responses generated by synergistic cells were significantly greater than the calculated sum (Sum) of L-amino acid and IMP responses. For non-synergistic cells, MIX responses were significantly smaller than the calculated sums of responses to L-amino acid and IMP. MIX responses of synergistic cells were also significantly greater than the MIX responses of non-synergistic cells. Numbers in parenthesis are the number of cells. One-way ANOVA followed by Bonferroni post hoc tests were used for statistical comparisons. ***P<0.0001, **P<0.001.

2.4.3 MIX-Responsive TSCs Respond Differently to IMP and L-Amino Acids:

To further differentiate L-amino acid response patterns, we analyzed TSC responses to individual L-amino acid set, i.e., IMP and L-amino acid with and without IMP. Each L-amino acid set consisted of one of the four L-amino acids (MPG, Ser, Arg, and Gln), IMP, and the MIX (the L-amino acid+IMP). Each set of MIX-responsive TSCs was subdivided according to their responsiveness to the individual components of the MIX. For the MPG set (Table 2.2), which consisted of MPG, IMP, and MPG+IMP stimuli, 133 of 170 (78%) TSCs responded to the MPG+IMP-MIX. Of these 133 cells, a subset of 23 (17%) TSCs responded only to the MIX, but not to MPG or IMP presented individually, and 100% (23 out of 23 cells) of those responses were synergistic. On the other hand, another subset of 20 out of the 133 (15%) MIX-responsive cells responded to MPG but not IMP, and 8 of those 20 (40%) cells exhibited synergistic responses. Furthermore, another subset of 45 of the 133 (34%) MPG+IMP-responsive cells responded to IMP but not to MPG alone. Of these 45 TSCs, 30 (66%) TSCs responded synergistically. Lastly, a different subset of 45 TSCs of 133 MPG+IMP-responsive cells also responded to both IMP and MPG when presented alone. Of these 45 cells, 26 (58%) cells demonstrated a synergistic response to the MIX (Table 2.2). Similar response patterns were also found for the other three L-amino acid (Ser, Arg, and Gln) sets (Table 2.2). In summary, some TSCs responded to a MIX of an L-amino acid+IMP, but only a subset of those MIX-responsive cells was synergistic. Moreover, MIX-responsive TSCs may or may not respond to the individual components of the MIX. Additionally, clustering of MIX-responsive TSCs by their response to individual stimuli showed that

almost all of the TSCs that did not respond to individual stimuli (i.e., L-amino acid or IMP), generated a synergistic response to the MIX.

Table2.2: Summary of WT MIX-responsive synergistic and non-synergistic TSCs.

MPG+IMP-MIX-Responsive TSCs

Calcium Response	**Synergistic**	**Non-Synergistic**	
YES = + ; No = -	Number of TSCs		**Total**
MPG - / IMP -	23	0	**23 (17)**
MPG - / IMP +	30	15	**45 (34)**
MPG + / IMP -	8	12	**20 (15)**
MPG + / IMP +	26	19	**45 (34)**
Total	**87 (65)**	**46 (35)**	**133**

Ser+IMP-MIX-Responsive TSCs

Calcium Response	**Synergistic**	**Non-Synergistic**	
YES = + ; No = -	Number of TSCs		**Total**
Ser - / IMP -	13	0	**13 (11)**
Ser - / IMP +	32	14	**46 (39)**
Ser + / IMP -	7	7	**14 (12)**
Ser + / IMP +	7	38	**45 (38)**
Total	**59 (50.0)**	**59 (50.0)**	**118**

Arg+IMP-MIX-Responsive TSCs

Calcium Response	**Synergistic**	**Non-Synergistic**	
YES = + ; No = -	Number of TSCs		**Total**
Arg - / IMP -	15	2	**17 (15)**
Arg - / IMP +	36	16	**52 (47)**
Arg + / IMP -	3	5	**8 (7)**
Arg + / IMP +	16	18	**34 (31)**

Total	**70 (63)**	**41 (37)**	**111**

	Gln+IMP-MIX-Responsive TSCs		
Calcium Response	**Synergistic**	**Non-Synergistic**	
YES = + ; No = -	Number of TSCs		**Total**
Gln - / IMP -	14	2	**16 (14)**
Gln - / IMP +	23	23	**46 (40)**
Gln + / IMP -	6	1	**7 (6)**
Gln + / IMP +	32	13	**45 (40)**
Total	**75 (65)**	**39 (34)**	**114**

MIX-responsive TSCs responded differently to the individual components of each L-amino acid set. Values are number of cells. Values in parenthesis are percentages. +, response; -, no response to the stimulus. Note: For 2 cells in the Arg set, and 2 cells in the Gln set, MIX responses were not synergistic even though IMP nor the L-amino acid alone elicited a response (their increases in Ca^{2+} were <5% of baseline). For these cells, the increases in Ca^{2+} in response to IMP, and L-amino acids individually were between 2.5% and 3% above baseline, and thus were not considered to be responses. However, the MIX elicited very small responses that were just above 5% of baseline Ca^{2+}, but not larger than the added sum of individual responses. Thus, these MIX responses were not synergistic. Since only around 2% of the cells showed this type of response, we did not include these cells in our discussion of synergy.

2.4.4 Synergistic and non-synergistic responses are mediated by different receptors:

In recent years, evaluation of taste cell transduction mechanisms has focused mostly on whether or not TSCs exhibit any Ca^{2+} increase in response to stimulation, whereas much less attention has been given to the intensity of these responses. Since changes in response intensity related to synergy are likely to take place through mechanisms within the signal transduction pathway, we compared the average Ca^{2+} responses to the individual stimulus components of the MIX of two groups of MIX-responsive cells: 1) non-synergistic and 2) synergistic cells. Interestingly, increases in intracellular Ca^{2+} in response to IMP were significantly smaller for synergistic cells compared to non-synergistic cells (Unpaired t-test; $P<0.0001$) (Fig. 2.3). Similarly, when stimulated with L-amino acids (MPG, Ser, or Arg), the intracellular Ca^{2+} responses to these L-amino acids were significantly smaller for synergistic cells compared to non-

synergistic MIX-responsive cells (One way ANOVA; P<0.0001) (Fig. 2.3). These response patterns suggest the involvement of more than one receptor. One possible scenario is that there are two types of receptors. One type of receptor may be involved in synergistic responses with no or small responses to individual components of the MIX. The second type of receptor may respond to the individual components of the MIX (L-amino acids or IMP), and any reaction to the MIX is solely a response to the components in the MIX.

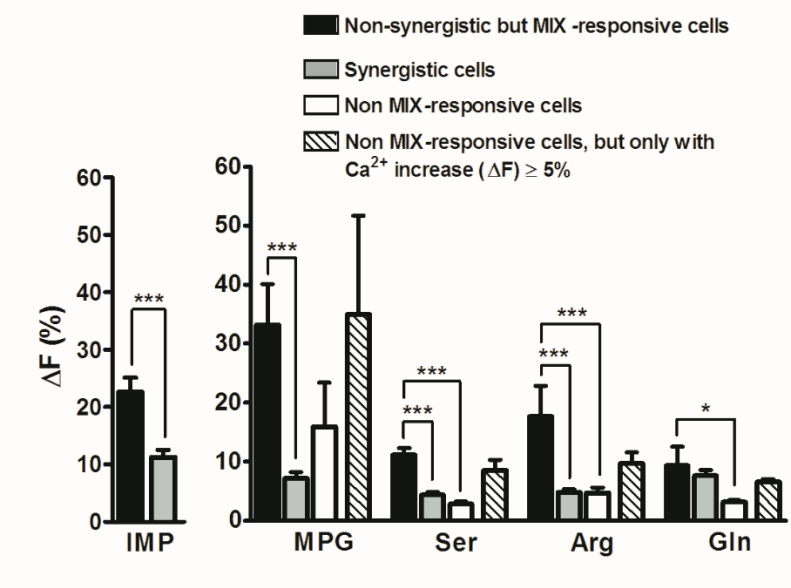

Figure 2.3: Ca^{2+} responses to L-amino acids and IMP in MIX-responsive and non-MIX-responsive cells.

Bars represent Mean±SEM Ca^{2+} responses. Average Ca^{2+} responses to the individual stimulus components of the MIX were compared for two groups of MIX-responsive cells: 1) non-synergistic and 2) synergistic cells. The increases in intracellular Ca^{2+} in response to IMP and the L-amino acids were significantly smaller for synergistic cells (gray bars) compared to non-synergistic (black bars) MIX-responsive cells. Cells that did not respond to the MIX but responded to an L-amino acid (non-MIX-responsive cells; white bars) presented alone also generated Ca^{2+} responses with a similar magnitude as those of MIX-responsive non-synergistic cells (gray bars). To eliminate bias by cells that may not have a receptor, only those cells with Ca^{2+} responses ($\Delta F \geq 5\%$) to L-amino acids were analyzed (see results). For these cells, Ca^{2+} responses to individual L-amino acids were not significantly different from Ca^{2+} responses of MIX-responsive, non-synergistic cells (patterned bars). Unpaired t-test (for IMP), and One-way ANOVA followed by Bonferroni post hoc t-test (for L-amino acids) were used for statistical comparison. ***P<0.0001, **P<0.001, *P<0.05.

Since synergistic TSCs had smaller responses to the individual components of a MIX than non-synergistic TSCs, we asked whether cells that do not respond to the MIX but respond to an L-amino acid (non-MIX-responsive cells) presented alone also generated Ca^{2+} responses with a similar magnitude as those of MIX-responsive non-synergistic cells. Surprisingly, for non-MIX-responsive cells, Ca^{2+} responses to individual L-amino acids were also significantly smaller than Ca^{2+} responses of MIX-responsive, non-synergistic cells (Fig. 2.3). Cells that responded to IMP but not to the MIX or to the individual L-amino acid of the MIX may not express any receptor for L-amino acid binding, thereby skewing the whole population of data towards null responses. To avoid this problem, we considered only cells with Ca^{2+} responses ($\Delta F \geq 5\%$) to L-amino acids. As expected, these responses were not significantly different from non-synergistic MIX-responsive cells (Fig. 2.3). This further supports the possibility that different receptors are probably involved in synergistic and non-synergistic responses.

2.4.5 Some TSCs were broadly tuned:

To further characterize the response-specificity of TSCs we tested additional TSCs to determine if they responded to L-amino acids as well as KCl, bitter and/or sweet, stimuli. In total, 135 isolated TSCs were tested with the mixture of L-amino acids (AA-MIX), sweet, and bitter stimuli. Of 135 cells, 13 (10%) cells responded to AA-MIX, 43 (31%) cells responded to bitter stimuli, and 26 (19%) cells responded to the sweet stimulus. The mean amplitude of Ca^{2+} increase (ΔF) for AA-MIX, bitter, and sweet stimuli were 40.26±15.13, 15.05±2.57, and 16.47±5.61 (Mean±SEM) above baseline, respectively. Of the 13 AA-MIX-responsive cells, 10 cells (77%) responded to the AA-

MIX only (Fig. 2.4A). The other 3 AA-MIX responsive TSCs also responded to sweet, bitter, or both of the stimuli (Fig. 2.4B, C, D). Additionally, we tested 9 of these TSCs with the high-K^+ solution to see if they were also responsive to high-K^+. Of these 9 cells, only 1 isolated cell (11%) responded to high-K^+ (Fig. 2.4C). This cell was also responsive to sweet stimuli. Tomchik et al. [46], proposed that this cell type may be a pre-synaptic cell. On the other hand, Dando and Roper suggested that this type of response may be the result of cell-to-cell communication between Type II and Type III cell [47]. However, in our case the recording was obtained from an isolated cell, thus the response elicited by this cell cannot be influenced by another cell. It should also be noted that none of the cells that responded only to AA-MIX, responded to high-K^+ solution

Although our focus was on the AA-MIX-responsive cells, we also evaluated these cells from the perspective of bitter and sweet responsiveness. Of the 43 bitter responsive cells, 24 cells (57%) responded to only bitter (Fig. 2.4E), and of 26 sweet responsive cells, 7 cells (27%) responded to only sweet stimuli (Fig. 2.4F). These data suggest that while some cells are narrowly tuned to a specific type of stimulus, at lease a small proportion cells are broadly tuned to multiple types of taste stimuli. Our data are in agreement with previous studies, which also reported the presence of broadly tuned and narrowly tuned TSCs [46, 48, 49].

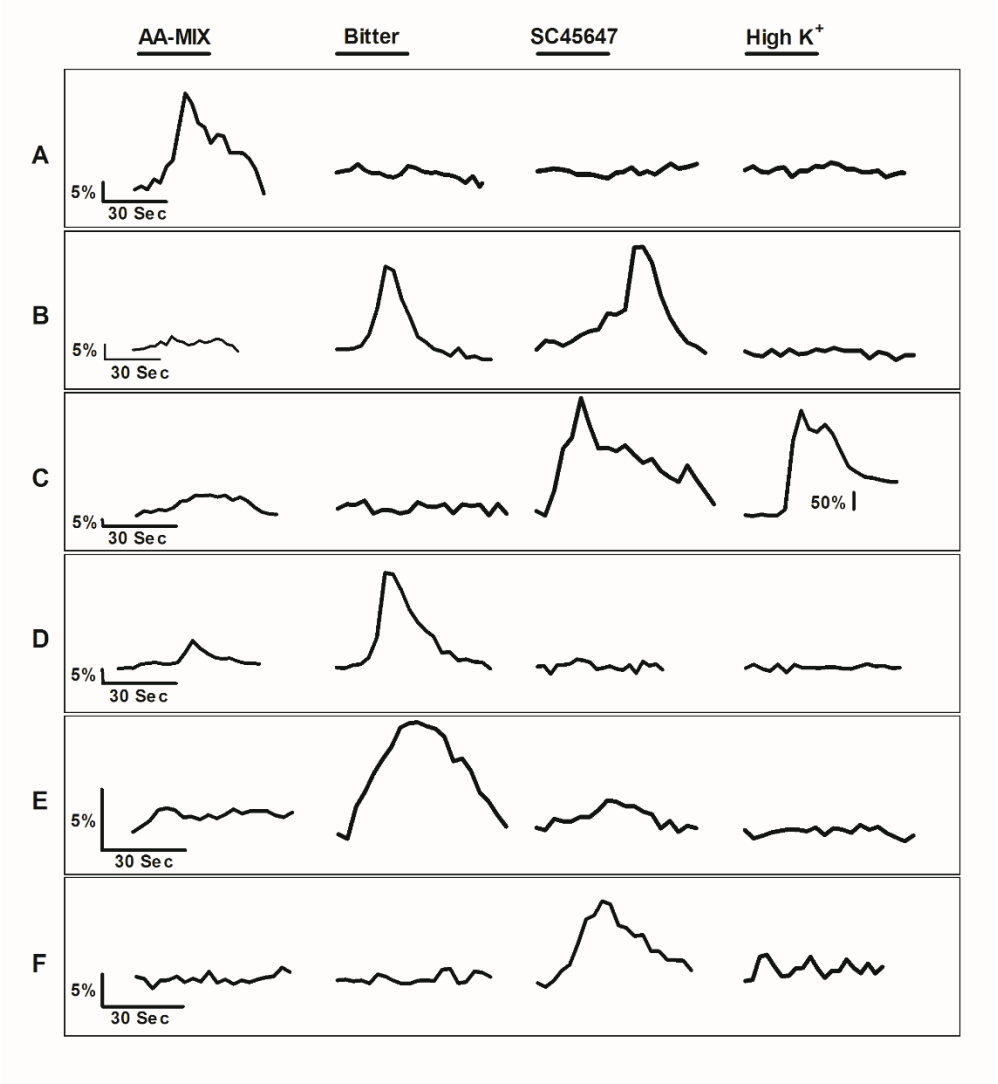

Figure 2.4: Representative Ca^{2+} responses elicited by TSCs during stimulation with 4 different stimuli.

Cells were stimulated with 4 different stimuli, L-amino acid MIX (AA-MIX) (MPG (10mM), Ser (20mM), Arg (10mM), and Gln (10 mM)), sweet (SC45647 (100µM)), bitter (denatonium (2mM) or cycloheximide (20µM)+denatonium (2mM)), and high-K^+ (65mM) solution. The bar above each stimulus trace represents the 30 s stimulus application period. [A] A TSC that responded only to AA-MIX. [B] A TSC that responded to bitter and sweet stimuli. [C] A TSC that responded to AA-MIX, sweet, and high-K^+ solution. AA-MIX elicited a very small increase in cytosolic Ca^{2+} compared to sweet stimulus. The high-K^+ solution elicited very large increase in cytosolic Ca^{2+}, suggesting the presence to voltage-gated calcium channels. Note: A different Y axis scale was used for the high-K^+ response as the high-K^+ elicited response was much larger compared to AA-MIX or sweet responses. [D] This TSC responded to both AA-MIX and bitter stimuli. AA-MIX elicited a very small increase in cytosolic Ca^{2+} compared to bitter stimulus. [E] This TSC responded only to bitter stimuli. [F] This TSC responded only to sweet stimuli.

2.4.6 TSCs from T1r3 KO mice responded to different L-amino acids:

The taste receptor T1r1+T1r3 heterodimer functions as an umami and L-amino acid receptor in mice. To determine if T1r3 is an obligatory component for L-amino acid responses, TSCs from circumvallate and foliate papillae of T1r3 KO mice were tested with the same stimuli used to test TSCs from WT mice. We screened a total of 320 TSCs of T1r3 KO mice for responses and successfully tested 154 cells with all 9 stimuli. Ca^{2+} responses were detected from 24 of 154 (16%) cells. As a control, we compared the high-K^{+} induced responses of TSCs of WT and T1r3 KO mice. The incidence and magnitude of these responses were not significantly different between WT and T1r3 KO mice (WT, n=20 out of 56 cells (36%); T1r3 KO, n=23 out of 61 cells (38%); Unpaired t-test, P=0.87).

TSCs from T1r3 KO mice not only responded to L-glutamate but also responded to other L-amino acids (Table 2.1; Fig. 2.5). Of the 24 responsive cells, MPG elicited an increase in Ca^{2+} of 14.6±4.9% (Mean±SEM) above baseline in 16 (67%) TSCs (Table 2.1; Fig. 2.5).Ser, Arg, and Gln also elicited responses in 24 (100%), 18 (75%), and 18 (75%) TSCs, respectively, although the populations of responsive cells were not identical. Like TSCs of WT mice, L-amino acids elicited a response in some but not all TSCs when presented with IMP (Table 2.1; Fig. 2.5). Additionally, IMP presented alone elicited Ca^{2+} responses in 14 of the 24 (58%) responsive TSCs. The magnitude of responses to IMP was 32.6%±6.2 (Mean±SEM). The results from TSCs of T1r3 KO mice lacking one component of the T1r umami receptor show that many of these TSCs are still capable of responding to different L-amino acids. Interestingly, when we compared the

percentage of responsive cells between WT and T1r3 KO mice, we found there were significantly fewer responsive cells for the T1r3 KO mice compared to the WT mice (Chi square test; $P<0.01$) but the proportion of TSCs that responded to each L-amino acid (except for MPG) was significantly larger for T1r3 KO mice (Chi square test; $P<0.05$). This may reflect the involvement of multiple receptors in the detection of L-amino acids. The absence of one receptor appears to decrease the number of L-amino acid responsive cells. However, the cells that are still capable of detecting an L-amino acids, are utilizing fewer receptor types and consequently making their responses more homogenous. This further supports the hypothesis that although T1r1+T1r3 receptor is involved in L-amino acid transduction, it is not the only receptor involved in L-amino acid taste.

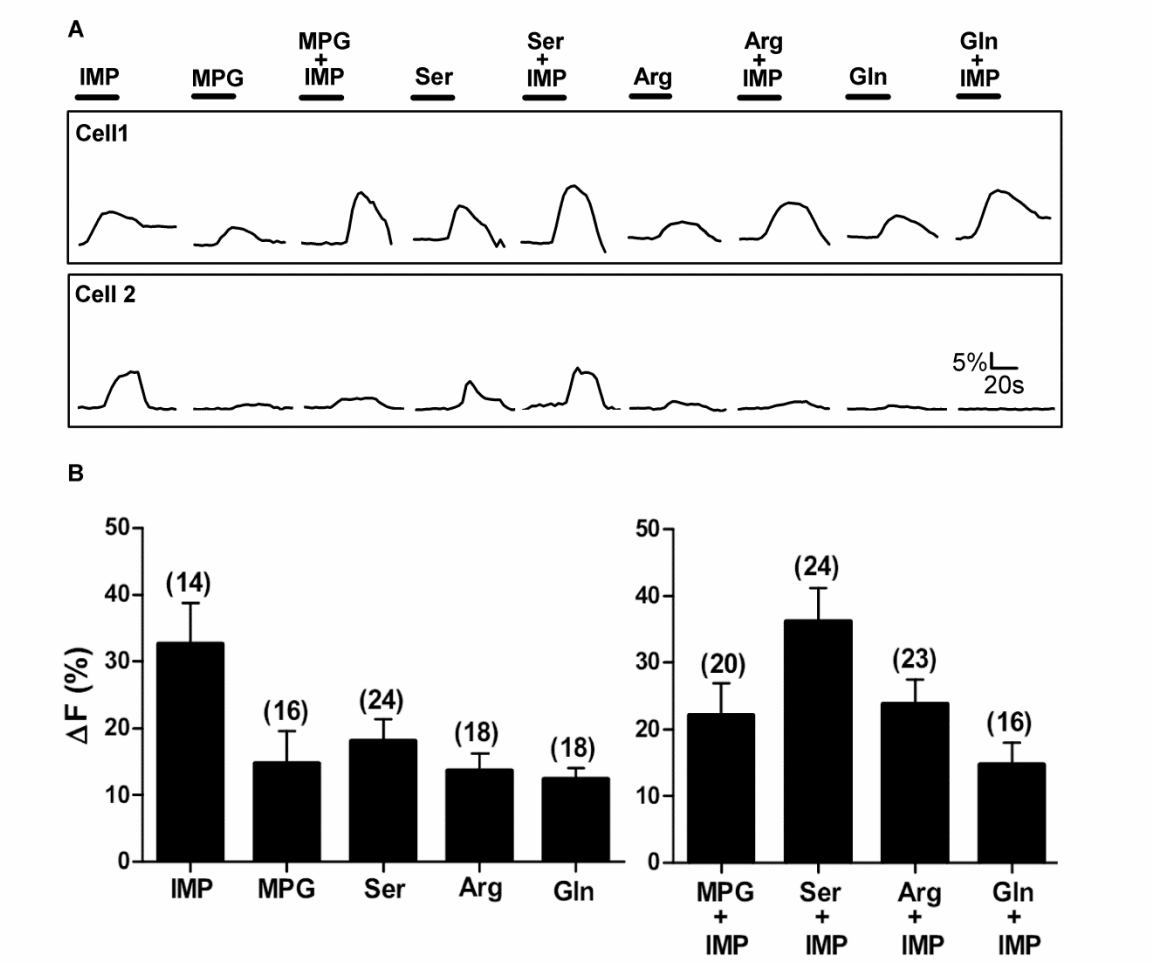

Figure 2.5: Representative Ca^{2+} responses of 2 TSCs from T1r3 KO mice.
Stimuli tested were IMP (1mM), 4 different L-amino acids (MPG (10mM), Ser (20mM), Arg (10mM), and Gln (10mM)), and L-amino acids with IMP. [A] Calcium responses of 2 sample TSCs. Each cell was tested with all 9 stimuli. The responses of these cells are examples of some of the different response patterns elicited by the array of stimuli. The bar above each stimulus trace represents the stimulus application time (30 sec). [B] Mean±SEM amplitude of Ca^{2+} increase (ΔF%) above baseline for responsive cells only, i.e. only cells with a change in baseline Ca^{2+}≥5%. Numbers in parenthesis are the number of cells.

1.3.6 Receptor(s) other than T1r1+T1r3 may be involved in synergistic responses:

We next analyzed responses for each L-amino acid set to determine if TSCs from T1r3 KO mice also showed any synergistic responses. For each L-amino acid set, a subset of MIX responses was greater than the sum of the responses to individual components of the MIX (Table 2.1; Fig. 2.6). Of 21 MPG+IMP-MIX-responsive cells, 9

(43%) cells exhibited synergistic responses. Likewise for Ser, Arg, and Gln sets 63%, 61%, and 13% of the MIX-responsive cells showed synergistic responses, respectively (Table 2.1). In addition, similar to WT responses, synergistic MIX responses of the KO mice were significantly greater than the calculated summed responses for MPG, Ser, and Arg set (One way ANOVA; $P<0.05$) (Fig. 2.6). We further compared the percentage of MIX-responsive cells between WT and T1r3 KO mice. The percentage of cells responding to the MIX for each L-amino acids were not different between WT and T1r3 KO mice (Chi square test; $P>0.05$). However, for the Ser and Arg sets, the percentages of synergistic cells were significantly greater in T1r3 KO mice (Chi square test; $P<0.05$). These results further suggest that receptor(s) other than T1r1+T1r3 heterodimer are involved in L-amino acid detection and that other receptors can also elicit synergistic responses.

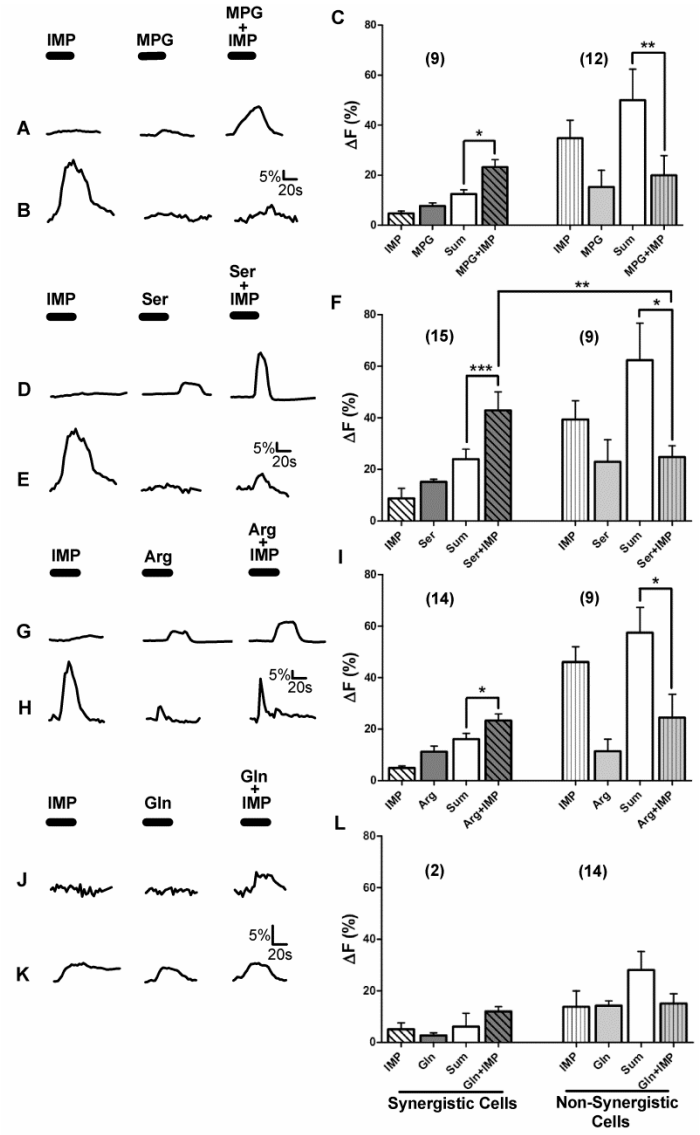

Figure 2.6: TSCs from T1r3 KO mice can also elicit synergistic responses.
Ca^{2+} responses of T1r3 KO TSCs to the four L-amino acid sets are shown. Each L-amino acid set consisted of one of the four L-amino acids (MPG (10mM), Ser (20mM), Arg (10mM), or Gln (10mM), respectively), IMP (1mM), and the MIX of L-amino acid+IMP. [A, D, G, J] Representative TSC responses when the magnitudes of MIX responses were greater than the summation of individual L-amino acid and IMP responses, i.e., the MIX responses were synergistic. [B, E, H, I] Representative TSC responses where the magnitudes of MIX responses were greater than or equal to the response of the L-amino acid or IMP individually, but not greater than the summation of the individual L-amino acid and IMP responses, i.e., the MIX responses were not synergistic. [C, F, I, L] Mean±SEM response for the L-amino acid sets exhibiting synergistic and non-synergistic responses. We were unable to conclude anything about Gln set as only 2 cells responded synergistically to Gln+IMP. Numbers in parenthesis are the number of cells. Mann Whitney test was used for statistical testing. ***P<0.001, **P<0.01, *P<0.05.

Clustering of MIX-responsive cells of WT mice to evaluate their responses to individual stimuli showed that almost 100% of the cells that did not respond to one or the other of the individual stimuli i.e., L-amino acid or IMP, responded synergistically to the MIX (Table 2.2). Interestingly, only three TSCs from T1r3 KO mice exhibited similar response patterns (2 for MPG, 1 for Gln set (Table 2.3)). Additionally, a more direct comparison of the synergistic responses by TSCs of WT and T1r3 KO mice revealed that the mean amplitude of responses elicited by T1r3 KO cells were significantly smaller for MPG+IMP, and Arg+IMP (Unpaired t-test; $P<0.05$; Fig. 2.7). However, Ser+IMP synergistic responses of T1r3 KO cells were not different from WT cells. Moreover, the mean amplitude of synergistic MIX responses by T1r3 KO cells for the Ser set was significantly greater than non-synergistic MIX responses (Fig. 2.6F). These data suggest that, while the T1r1+T1r3 heterodimer is important for synergistic responses, other receptors also play a role in eliciting synergistic responses.

Table1.3: Summary of T1r3 KO MIX-responsive synergistic and non-synergistic TSCs.

	MPG+IMP-MIX-Responsive TSCs		
Calcium Response	**Synergistic**	**Non-Synergistic**	
YES = + ; No= -	Number of TSCs		**Total**
MPG - / IMP -	1	0	**1 (5)**
MPG - / IMP +	0	4	**4 (19)**
MPG + / IMP -	5	2	**7 (33)**
MPG + / IMP +	3	6	**9 (43)**
Total	**9 (43)**	**12 (57)**	**21**

	Ser+IMP-MIX-Responsive TSCs	
Calcium Response	**Synergistic**	**Non-Synergistic**

YES = + ; No = -	Number of TSCs		**Total**
Ser - / IMP -	0	0	**0 (0)**
Ser - / IMP +	0	0	**0 (0)**
Ser + / IMP -	9	1	**10 (42)**
Ser + / IMP +	6	8	**14 (58)**
Total	**15 (63)**	**9 (37)**	**24**

Arg+IMP-MIX-Responsive TSCs

Calcium Response	**Synergistic**	**Non-Synergistic**	
YES = + ; No = -	Number of TSCs		**Total**
Arg - / IMP -	0	0	**0 (0)**
Arg - / IMP +	1	4	**5 (22)**
Arg + / IMP -	9	0	**9 (39)**
Arg + / IMP +	4	5	**9 (39)**
Total	**14 (61)**	**9 (39)**	**23**

Gln+IMP-MIX-Responsive TSCs

Calcium Response	**Synergistic**	**Non-Synergistic**	
YES = + ; No = -	Number of TSCs		**Total**
Gln - / IMP -	1	0	**1 (6)**
Gln - / IMP +	1	0	**1 (6)**
Gln + / IMP -	0	8	**8 (50)**
Gln + / IMP +	0	6	**6 (38)**
Total	**2 (12)**	**14 (88)**	**16**

Values are number of cells. Values in parenthesis are percentages. +, response; -, no response to the stimulus.

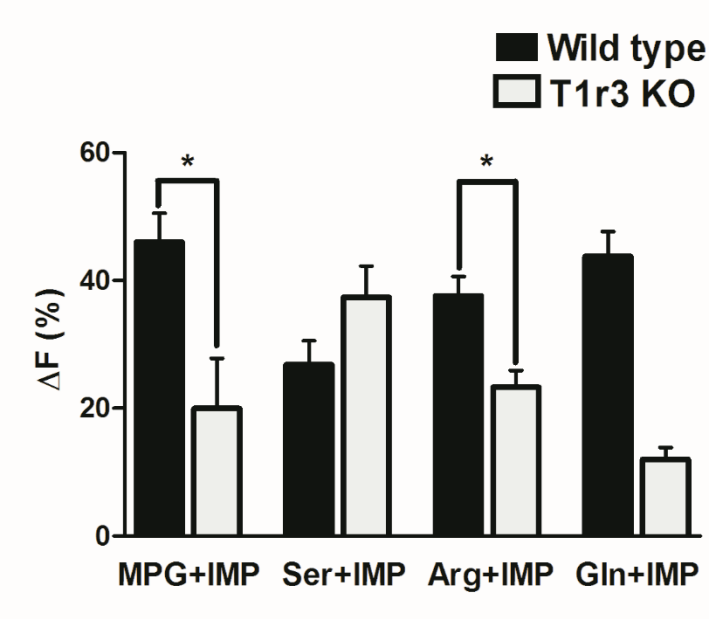

Figure 2.7: Comparison of amplitude of synergistic responses between WT and T1r3 KO mice. Bars represent Mean±SEM for synergistic responses (i.e., responses to the MIX that were greater than the sum of individual responses) of TSCs from WT and T1r3 KO mice. The amplitude of responses elicited by MPG+IMP and Arg+IMP was significantly smaller in T1r3 KO cells than those of WT cells (Unpaired t-test). *P<0.05.

2.4.7 TSCs in Clusters:

In our experiments isolated TSCs and TSCs in clusters were examined. TSCs in clusters are more stable, and stayed healthy for longer periods, thus allowing longer imaging time. One drawback of using clusters of cells, however, can be indirect activation of some TSCs in direct contact with another cell. There are two possible models through which an L-amino acid taste stimulus can activate a second-messenger-dependent (inositol triphosphate, IP_3) calcium wave that propagates between adjacent TSCs: (1) IP_3 may traverses through gap junctions and initiates the release of intracellular calcium stores in neighboring Type II or III cells (as found in different cellular systems) [50], (2) a Type II cell releases ATP that acts on a Type III (presynaptic) cell to increase intracellular Ca^{2+} which e.g., could lead to release of neurotransmitter stored in vesicles

[47, 51-53]. In either case, adjacent cells could generate similar response patterns within our imaging procedures. To identify cells that might be responding indirectly, we analyzed the response patterns of cells adjacent to each other. Of the 170 cells, 19% of the cells were close enough together for one of the cells to potentially be activated by another. Some of the adjacent cells in a cluster generated Ca^{2+} response patterns with comparable time courses and amplitudes. For example, cells 1 and 2 in Fig. 2.8A responded to all 9 stimuli with very similar increases in intracellular Ca^{2+}. Furthermore, both cells generated synergistic responses to all four L-amino acid+IMP MIXes (Fig. 2.8A). Similarly, cell 1 and cell 2 in Fig. 2.8B generated comparable Ca^{2+} response patterns to all 9 stimuli but in this case the response amplitude of cell 2 was around 50% of cell 1, and their responses were non-synergistic to all the four L-amino acid+IMP MIXes. In another instance, while one cell generated a synergistic response to the L-amino acids+IMP MIXes, the responses of the adjacent cell were non-synergistic (Fig. 2.8C). Thus, even though adjacent cells might appear to have similar response patterns to a stimulus, they often did not generate responses with the same temporal and intensity characteristics. In other cases, adjacent cells did not have comparable response patterns. For example, Fig. 2.8D shows Ca^{2+} responses of a pair of cells that had quite different response patterns to the 9 stimulus compounds. Conversely, none of the response patterns of adjacent cells were unique when compared to those of isolated cells, but rather they generated responses that were generally indistinguishable from isolated TSCs. When we eliminated 50% of these adjacent cells (assuming half of the cells responded to L-amino acid stimuli, and the rest demonstrated a Ca^{2+} increase due to indirect activation) from their respective data sets, there was a small reduction in the number of cells per group but

no change in the overall findings from our experiments. Similar observations were made for TSCs of T1r3 KO mice.

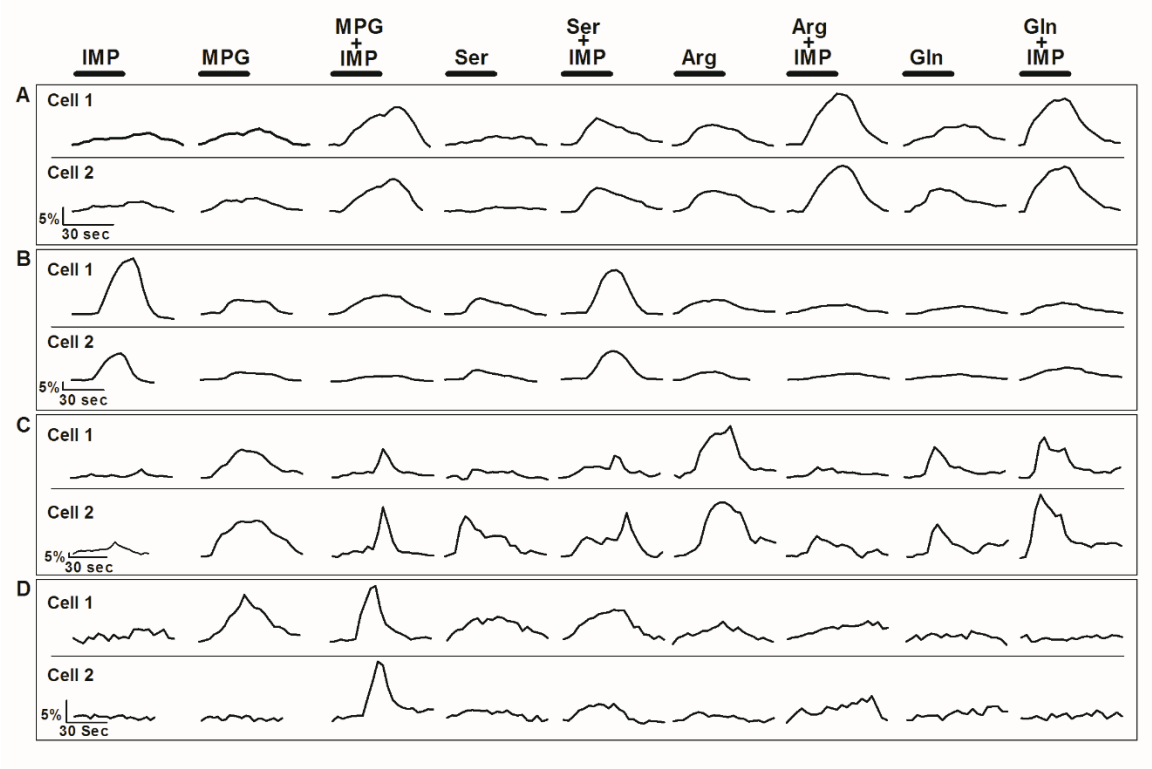

Figure 2.8: Representative Ca^{2+} responses of adjacent WT TSCs in clusters.
Cells were stimulated with 9 different stimuli: IMP (1mM) 4 different L-amino acids (MPG (10mM), Ser (20mM), Arg (10mM), and Gln (10mM)), and L-amino acid+IMP. The bar above each stimulus trace represents the stimulus application time (30 sec). [A] Cells 1 and 2, adjacent TSCs in a cluster, generated Ca^{2+}responses similar in temporal and amplitude to each stimulus, including synergistic responses to all the L-amino acid+IMP MIXes. [B] These cells were adjacent TSCs in a cluster, exhibited similar non-synergistic Ca^{2+} responses to all the L-amino acid+IMP MIXes, but the response amplitudes of cell 2 was 50% of cell 1. [C] Cells 1 and 2 were adjacent TSCs in a cluster that responded to the same stimuli. However, one cell responded synergistically to the MIXes whereas the other cell responded non-synergistically. [D] Cells 1 and 2 were adjacent TSCs. Each cell exhibited different response patterns to the array of test stimuli.

2.5 Discussion

Although much is known about the receptor systems and transduction mechanisms involved in the detection of L-glutamate, the prototypical umami L-amino acid, the mechanisms for detecting other L-amino acids are not well understood. In

general, detection of L-amino acids by the taste system has been linked closely to detection of umami stimuli through a presumed common taste receptor and through interactions with 5'-ribonucleotides. The impetus for this connection was strengthened considerably by the discovery that most L-amino acids appear to be able to activate T1r1+T1r3 receptors in HEK cells, especially when they were mixed with IMP, and by a number of subsequent studies [23-25]. There is growing evidence, however, that other receptors may contribute to umami taste sensations, including taste-mGluR4, taste-mGluR1 and possibly others [20, 29, 30, 54, 55]. If a single receptor is responsible for detecting all L-amino acids, then they should induce responses in the same TSCs and elicit the same or similar taste qualities, including synergistic responses when they are mixed with 5'-ribonucleotides. However, conditioned taste aversion and discrimination studies in rats and mice have shown that L-amino acids do not elicit the same taste qualities [33, 55]. Similarly, psychophysical studies have shown that humans perceive Ser and Gln as sweet at low concentrations and umami at high concentrations but they perceive Arg as bitter [34, 35]. The results of these experiments appear to be more in consistent with the hypothesis that the sensations elicited by each L-amino acid may be the product of the combined contributions of multiple L-amino acid receptors rather than a single receptor.

To more directly evaluate this hypothesis, Ca^{2+} imaging of isolated mouse TSCs and taste cell clusters were performed with a panel of four L-amino acids (MPG, Ser, Arg, and Gln). Our aim was to determine if single TSCs are responsive to all or a subset of L-amino acids and whether these cells showed evidence of synergy when mixed with

IMP. We observed a wide range of response patterns of single TSCs tested in isolation or in clusters with all 9 stimuli but only a few TSCs responded to all 9 stimuli (10 out of 170 cells; 6%). Mixing IMP with L-amino acids elicited synergy for all 4 L-amino acids tested, but not every MIX-responsive cells responded synergistically. In addition, TSCs from T1r3 KO mice showed response patterns comparable to those of WT mice.

Since we bath applied stimuli, some responses may be due to activation of glutamate receptors expressed in the basolateral membrane of the cells. Several neurotransmitters have been proposed to function in the taste buds, including glutamate, serotonin, gamma amino butyric acid, norepinephrine, acetylcholine, ATP, CCK, and neuropeptide Y [57–74], but only serotonin, norepinephrine, and ATP have been unambiguously identified and shown to be released in response to stimulation [60, 61, 64–66]. Studies suggesting glutamate as a potential neurotransmitter are mainly based upon the expression of ionotropic and metabotropic glutamate receptors in the lingual tissue, including taste buds, and the expression of glutamate transporter GLAST in Type I taste cells [20, 21, 47, 58, 67, 72, 75]. Vandenbeuch et al. [72] found vesicular glutamate transporters, VGLUT1 and 2, expressed in the afferent nerve fibers, but not in taste bud cells. Thus glutamate may be released by afferent nerve fibers, and may modulate taste function [58, 62, 72, 75]. However, to date there is no report that directly shows the release of glutamate in taste buds, or any modulatory function of glutamate in the taste buds. Nevertheless, we realize this may be a limitation of our protocol, and that there may be glutamate receptors at the basolateral end of the TSCs. If so, some of the responses will not be normal taste responses.

2.5.1 Multiple receptors and/or transduction mechanisms are involved in L-amino acids and IMP taste

In our experiments, TSCs of WT mice often responded to more than one L-amino acid, but not all L-amino acids elicited a response in the same TSC (Table 2.1; Fig. 2.1). Some but not all L-amino acid responsive cells responded to glutamate, the prototypical umami taste stimulus. When TSCs were stimulated with the MIX of an L-amino acid and IMP, a diverse array of response patterns were found. For example, 1) the MIX for each L-amino acid elicited a response in some TSCs, 2) cells that responded to the individual components of a MIX did not always respond when the MIX was applied, and 3) a subset of MIX-responsive TSCs did not respond to the individual components of the MIX (Table 2.2; Fig. 2.2). Prior recording studies of the chorda tympani (CT) and glossopharyngeal (GL) nerve fibers of rats and mice have shown that 5'-ribonucleotides or glutamate alone can elicit measurable responses. Although many nerve fibers responded to IMP, GMP, and glutamate, some responded to only 5'-ribonucleotides or glutamate. In addition, only a subset of fiber responses to a glutamate+5' ribonucleotide-MIX was synergistic [44, 76-82]. Similar response patterns were also seen in patch clamp recordings and Ca^{2+} imaging experiments. Lin et al. [44] using taste cells isolated from rat fungiform papillae showed that a subset of TSCs responded to glutamate or GMP alone, and only a subset of glutamate+GMP-MIX-responsive cells showed synergy. Further, behavioral discrimination studies have shown that rats could positivity distinguish between MSG and IMP or GMP, suggesting that MSG and these 5' ribonucleotides possess at least some unique taste qualities [83]. Our Ca^{2+} imaging results

are consistent with these previous studies and suggests that TSCs may respond to IMP and the L-amino acids with different receptors and/or transduction pathways.

Our findings with isolated or clustered TSCs did not recapitulate response trends seen withT1r1+T1r3 heterodimer expressing HEK cells [24]. These HEK cells showed no Ca^{2+} increase when stimulated with the prototypical umami compound MSG (50mM). However, in our study 46% of the responsive TSCs responded to 10mM MPG presented alone. These differences in cellular responses might be due to the experimental model (*in vivo* versus *in vitro*) in which the receptor heterodimer is expressed, but it is also plausible that receptors other than T1r1+T1r3 are involved in L-amino acids and IMP detection.

2.5.2 In posterior tongue, receptor(s) other than T1r1+T1r3 can generate synergistic responses:

Comparison of TSCs from WT and T1r3 KO mice revealed some very interesting findings, especially when IMP was a part of the stimulus solution. Synergy between 5'-ribonucleotides and glutamate is a defining characteristic of umami taste. In HEK cells expressing T1r1+T1r3, several L-amino acids exhibited this synergistic characteristic in the presence of IMP [24], often responding to an L-amino acid only when IMP was present. A previous study reported that some TSCs responded synergistically to glutamate+IMP but had only small or no responses to glutamate or GMP alone [44]. Our analysis of the WT TSC responses to the 9 stimuli (IMP, 4 L-amino acids, with and without IMP) revealed an interesting cluster of 40 (24%) cells which responded to L-amino acids only in presence of IMP, and showed little or no response to IMP alone. We

also identified a subset of synergistic WT cells that had no detectable response to the individual components of the MIX for all four L-amino acids tested. This resulted in a noticeable increase in the number of cells responsive to L-amino acids in presence of IMP (Table 2.1). Interestingly, we did not find any such response patterns for TSCs of T1r3 KO mice. In the WT experiments, all 4 L-amino acid sets elicited synergistic MIX responses that had significantly higher peak amplitudes than non-synergistic MIX responses (Fig. 2.2C, F, I, L). In contrast, T1r3 KO cells responded synergistically to the MIXes but the responses to 2 of the 3 sets were not significantly larger than the non-synergistic MIX responses (Ser+IMP was the exception) (Fig. 2.6C, F, I). We were unable to conclude anything about the Gln set, as only 2 cells responded synergistically to Gln+IMP. These findings suggest that there is a different response mechanism that is dependent upon the L-amino acid ligand. This conclusion is further supported by synergistic responses of T1r3 KO cells. The responses of these cells to MPG+IMP and Arg+IMP-MIXes were significantly smaller than responses of WT cells (Fig. 2.7), whereas responses to Ser+IMP were not different from WT TSCs (Fig. 2.7). Collectively, these results suggest that although the T1r1+T1r3 heterodimer plays an important role in generating synergistic responses to these L-amino acids, it is not the only receptor capable of eliciting a synergistic response.

The characteristics of single fiber and whole-nerve responses to stimulation by L-amino acids vary considerably between CT and GL nerves [78, 80]. Ninomiya and colleagues identified taste fibers in the mouse GL nerve that responded to umami compound MSG, including fibers showing synergistic responses to MSG in the presence

of 0.5mM GMP [84, 85]. The importance of the GL nerve in umami taste was further established when Ninomiya and Funakoshi showed that mice with bilateral section of the GL nerve could not discriminate between MSG and NaCl [84, 85]. Moreover, the posterior part of the tongue is more sensitive to umami than the anterior tongue [86, 87]. On the other hand, whole nerve responses to umami are much greater in the CT than in the GL and synergy is detectable in the response of the CT but not the GL [88]. In addition, the sweet taste inhibiting peptide gurmarin inhibited the umami signal and synergistic responses preferentially in the CT of C57BL/6J mice, but had no detectible effect on GL nerve recordings. Thus there may be a different set and/or proportion of receptors that elicit umami taste in the posterior portion of the tongue. Even though whole nerve studies found minimal synergistic responses from the posterior portion of the tongue, in this study we found a heterogeneous group of cells in circumvallate and foliate taste buds that showed synergistic responses to L-amino acids and IMP stimuli.

Recently the Venus fly trap domain of the T1r1 subunit of the T1r1+T1r3 heterodimer was proposed to be critical for umami synergism [89, 90]. We found an unexpectedly high proportion of TSCs from T1r3 KO mice, like those of WT mice, responded not only to L-glutamate but also to other L-amino acids, with or without IMP. Some of these KO cells also exhibited synergistic responses when L-amino acids were mixed with IMP (Table 2.1; Fig. 2.2). The combination of these findings suggests that there is likely another receptor at least in the posterior region of the tongue that is capable of eliciting synergistic responses. In circumvallate papillae, the majority of the T1r3 expressing cells also express T1r1, but only around 50% of the T1r1 expressing cells co-

express T1r3 [91]. Thus it is possible that T1r1 may homodimerize or form heterodimers with other receptors, but at present there is little evidence supporting this possibility. Additionally, the expression of the T1r1+T1r3 heterodimer is not uniform throughout the different taste papillae and is lower in the posterior portion of the tongue [91–96]. Taste-mGluR4 has been proposed to be an alternate receptor involved in umami taste. The action of L-AP4, an agonist of taste-mGluR4, has been shown to be enhanced in the presence of IMP [96, 97]. Thus, taste-mGluR4 alone or with other receptor complexes may be involved in mediating synergistic responses.

2.5.3 IMP alone elicits responses in TSCs:

Our finding that IMP alone can elicit a response in a large number of TSCs was surprising since Lin et al. [44] reported finding far fewer fungiform TSCs in the rat that responded to GMP. This might be due to the difference in the species and the papillae from which the cells were isolated. However, the receptor involved in the detection of 5'-ribonucleotides remains unclear. An IMP binding site has been proposed to be located in the N-terminal domain of the T1r1 subunit [90], but the lack of IMP-induced Ca^{2+} responses in HEK cells expressing T1r1 or the heterodimer T1r1+T1r3 raises questions about its role as an IMP receptor. In our study, there was no difference between WT and T1r3 KO mice in the percentage of IMP responding cells. IMP responses by TSCs of T1r3 KO mice further support the hypothesis that receptor(s) other than the T1r1+T1r3 heterodimer can be activated by IMP. Studies with GPCRs and their agonists have shown that a single compound can act as either an agonist or an allosteric modulator, depending on receptor binding [98-100]. It is possible that IMP might act as either an agonist where

IMP itself can elicit significant Ca^{2+} responses without enhancing the response to another substance such as an L-amino acid, or as an allosteric modulator where it facilitates L-amino acid responses such as when IMP induces a synergistic response. Thus, it is possible that synergistic responses elicited by L-amino acids in presence of IMP are mediated primarily but not exclusively by the T1r1+T1r3 heterodimer, where L-amino acids act as umami compounds and IMP acts as an allosteric modulator. Other TSCs may have receptors that mediate non-synergistic responses to individual L-amino acids or IMP.

Although the specific receptors involved in detection of L-amino acids and IMP cannot be identified from these data, there may be several possibilities. Besides the T1r1+T1r3 heterodimer, it seems likely that mGluR receptors, including brain and truncated taste versions of mGluR4 and mGluR1 and possibly mGluR2 and mGluR3, may be potential candidates [20, 29-32, 54, 55]. Recently, the Ca^{2+} sensor CaSR and the class C GPCR, GPRC6A, which can also detect L-amino acids, were localized to Type I and III taste cells [101, 102]. However, whether these Ca^{2+} sensors can generate synergistic responses is yet to be determined. One very intriguing possibility is that taste-mGluR4 or some other receptor may form a complex with each other or with T1rs that responds to L-amino acids as well as generate synergistic responses. However, further investigation is needed to examine this hypothesis.

2.6 Conclusions

In summary, we report for the first time the response patterns of single TSCs to IMP and four L-amino acids (from different classes) with and without IMP. Our data

strongly suggest that, in addition to T1r1+T1r3, one or more receptor(s) other than T1rs contribute to the tastes of IMP and L-amino acids, as well as to synergistic interactions between IMP and L-amino acids. In particular, using Ca^{2+} imaging we showed that response patterns elicited by L-amino acids varied significantly across isolated TSCs. IMP and all four L-amino acids elicited Ca^{2+} responses in TSCs, although each cell typically responded to multiple, but not all L-amino acids. Moreover, in the presence of IMP, L-amino acids other than glutamate were able to elicit synergistic responses. Along with its role in synergism, IMP alone was capable of eliciting Ca^{2+} responses in TSCs of WT and T1r3 KO mice. We also found that TSCs from T1r3 KO mice can respond to L-amino acids and at least some are capable of synergistic responses in the presence of IMP. These results suggest that multiple receptors are involved in IMP and L-amino acid detection, as well as in generating synergistic responses.

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2.8 References

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Chapter 3 : METABOTROPIC GLUTAMATE RECEPTORS ARE INVOLVED IN THE DETECTION OF IMP AND L-AMINO ACIDS BY MOUSE TASTE SENSORY CELLS

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3.1 Abstract

G-protein coupled receptors are thought to be involved in the detection of umami and L-amino acid taste. These include the heterodimer T1r1+T1r3, taste and brain variants of mGluR4 and mGluR1, and calcium sensors. While several studies suggest T1r1+T1r3 is a broadly tuned L-amino acid receptor, little is known about the function of mGluRs in L-amino acid taste transduction. Calcium imaging of isolated taste sensory cells (TSCs) of T1r3-GFP and T1r3 knock-out (T1r3 KO) mice was performed using the ratiometric dye Fura 2 AM to investigate the role of different mGluRs in detecting various L-amino acids and inosine 5' monophosphate (IMP). Using agonists selective for various mGluRs such as (*RS*)-3, 5-dihydroxyphenylglycine (DHPG) (an mGluR1 agonist) and L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) (an mGluR4 agonist), we evaluated TSCs to determine if they might respond to these agonists, IMP, and three L-amino acids (monopotassium L-glutamate, L-serine and L-arginine). Additionally, we used selective antagonists against different mGluRs such as (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA) (an mGluR1 antagonist), and (*RS*)-α-methylserine-*O*-phosphate (MSOP) (an mGluR4 antagonist) to determine if they can block responses elicited by these L-amino acids and IMP. We found that L-amino acid- and IMP-responsive cells also responded to each agonist. Antagonists for mGluR4 and mGluR1 significantly blocked the responses elicited by IMP and each of the L-amino acids. Collectively, these data provide evidence for the involvement of taste and brain variants of mGluR1 and 4 in L-amino acid and IMP taste responses in mice, and support the concept that multiple receptors contribute to IMP and L-amino acid taste.

Key Words: L-amino acids, IMP, mGluR1, mGluR4, Taste, T1r3 knockout mice

Abbreviations: TSC, Taste Sensory Cells; MPG, Monopotassium glutamate; MSG, monosodium glutamate; Ser, L-Serine; Arg, L-Arginine; IMP, inosine 5' monophosphate; GMP, guanosine 5' monophosphate; mGluR, metabotropic glutamate receptor; iGluR, ionotropic glutamate receptor; HEK cells, Human Embryonic Kidney cells; GFP, Green fluorescent protein; GPCR, G-protein couple receptor; L-AP4, L-(+)-2-amino-4-phosphonobutyrate; CPPG, (RS)-α-cyclopropyl-4-phosphonophenylglycine; SC45647, 2-[[[[4-(aminomethyl)phenyl]amino]-[[(1*R*)-1-phenylethyl]amino]methyl]amino]ethane-1,1-diol; DHPG, (*S*)-3,5-Dihydroxyphenylglycine; CNQX, Cyano-7-nitroquinoxaline-2,3-dione; D-AP5, D-(-)-2-Amino-5-phosphonopentanoic acid; AIDA, (*RS*)-1-Aminoindan-1,5-dicarboxylic acid; MSOP, (*RS*)-α-Methylserine-*O*-phosphate, T1R1, Taste receptor type 1 member 1; T1R3, Taste receptor type 1 member 3; NMDA, *N*-Methyl-D-aspartic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, Analysis of variance. CTA, Conditioned taste aversion.

3.2 Introduction

Animals, including humans, must ingest dietary proteins composed of 20 different L-amino acids either of animal or plant origin. Detection of amino acids and protein in foods is thought to be linked to a unique basic taste called umami, a Japanese term roughly translated as "savory" (Ikeda, 2002). The L- amino acid glutamate is the prototypical compound representative of umami. Certain 5' ribonucleotide such as inosine 5' monophosphate (IMP), and guanosine 5' monophosphate (GMP) also have an umami taste (Kodama, 1913; Kuninaka, 1960; Yamamoto et al., 1991; Wifall et al., 2007). One interesting characteristic of umami taste is the remarkable synergistic enhancement of glutamate signaling when combined with IMP or GMP (Kuninaka et al., 1964; Tamaguchi and Kimizuka, 1979; Maga, 1983; Hiji and Sato, 1996a, b; Yamaguchi and Ninomiya, 2000). In addition to increasing the palatability of food, umami compounds can also cause vagal efferent activation and salivary flow responses that help in mastication and digestion of food (Bellisle et al., 1991; Niijima 2000; Hodson and Linden, 2006; Bellisle, 2008). Recent research has shown that fortification of meals with an appropriate amount of MSG may improve food intake and therefore has potential for improving the nutritional status and quality of life in elderly and nutritionally deficient patients (Schiffman, 2000; Toyama et al., 2008; Yamamoto et al., 2009). Thus, understanding the receptors and transduction pathways that mediate umami taste and potentially the tastes of L-amino acids in general could be beneficial in regulating ingestive behavior that contributes to good health.

Recent molecular, physiological, and behavioral studies have provided evidence of multiple candidate G-protein coupled receptors (GPCR) in umami taste. The first candidate taste receptor to be identified were two variants of the metabotropic glutamate receptor 4 (mGluR4), taste-mGluR4 (Chaudhari et al., 1996, 2000) and brain-mGluR4 (Toyono et al., 2002). When cloned, the taste-mGluR4 was found to be missing more than 50% of the N-terminal region compared to the brain-variant of mGluR4 (Chaudhari et al., 2000). Like the better known brain-mGluR4, the truncated taste variant also responds to glutamate and the group III selective mGluR agonist L-(+)-2-amino-4-phosphonobutyrate (L-AP4) (Chaudhari et al., 1996; Hayashi et al., 1996; Bigiani et al., 1997), although the affinity of taste-mGluR4 for glutamate and L-AP4 is more than 100 times lower than that of brain-type receptors (Chaudhari et al., 1996, 2000). Later a heterodimer receptor, T1r1+T1r3, was identified and is now one of the most firmly established umami receptor candidate (Li et al., 2002; Nelson et al., 2002). In heterologous expression systems (HEK cells), the human T1R1+T1R3 heterodimer is activated by glutamate, whereas mouse T1r1+T1r3 is activated by several amino acids, but in many cases only when L-amino acid is presented with IMP (Nelson et al., 2002). Further evidence for the role of T1r1+T1r3 as an umami taste receptor comes from studies with knockout (KO) mice in which the *Tas1r1* or *Tas1r3* gene was selectively eliminated (Damak et al., 2003; Zhao et al., 2003; Kusuhara et al., 2013). One KO mice model, having a deletion of the N-terminal extracellular domain but not the seven transmembrane helices of the gene (for *Tas1r3* gene) or vice versa (for *Tas1r1* gene) showed that mice lacking these receptors lose all ability to respond to umami stimuli , thus arguing that T1r1+T1r3 is the only umami receptor (Zhao et al., 2003). However,

other studies using an independently derived T1r1 and T1r3 receptor KO mouse model lacking the complete coding sequence of *Tas1r1* or *Tas1r3* gene found only partial taste loss for umami taste, evidence arguing that other receptors can also contribute to umami taste (Damak et al., 2003; Delay et al., 2006; Kusuhara et al., 2013).

Subsequently, additional candidate umami receptors have been identified in the oral cavity, including full-length variants of mGluR1 (Toyono et al., 2003) as well as a truncated variant of mGluR1 (taste-mGluR1) (San Gabriel et al., 2005, 2009a; Nakashima et al., 2012). Like taste-mGluR4, the truncated taste-mGluR1 also lacks much of the N-terminal extracellular domain and has more than 100-fold lower affinity for glutamate than does the brain-variant of the receptor (San Gabriel et al., 2005, 2009a). The full-length brain-mGluR1 and brain-mGluR4 are now known to be expressed in a subset of taste cells in fungiform, foliate and circumvallate papillae in rats in contrast to the truncated taste variants of mGluR1 and mGluR4 which are mainly expressed in the foliate and circumvallate papillae. Behavioral, nerve recording, and calcium imaging studies with rodents have shown that antagonists for mGluR4 and mGluR1 can selectively block or reduce responses elicited by glutamate (Nakashima et al., 2001; Yasuo et al., 2001; Eschle et al., 2009; Yoshida et al., 2009; Kusuhara et al., 2013).

Even though these studies suggest a role for mGluR4s and mGluR1s in the detection of glutamate, little is known about the involvement of mGluRs in detection of other L-amino acids and IMP. Similar to glutamate, other L-amino acids and IMP are present in free form in food, and thus can be detected by receptors expressed in the taste sensory cells (TSCs). The heterodimer T1r1+T1r3 is considered a broadly tuned L-amino

acid receptor, but in HEK cells it does not response to glutamate and IMP individually (Nelson et al., 2002). Rodents as well as humans can detect the taste of several L-amino acids and IMP. Previously we showed that TSCs of mice lacking the T1r3 receptor generate responses elicited by IMP and several L-amino acids, suggesting the involvement of multiple receptors in L-amino acids and IMP tastes (Pal Choudhuri et al., 2015). Moreover, an mGluR4 antagonist, (RS)-α-cyclopropyl-4-phosphonophenylglycine (CPPG), altered the degree of generalization of a conditioned taste aversion (CTA) between monosodium glutamate (MSG) and L-serine (Ser) and L-arginine (Arg) but not glycine in rats, suggesting that CPPG is capable of altering the taste qualities of these L-amino acids (Eschle et al., 2009). Therefore the question remains which receptor(s) is/are involved in the detection of residual L-amino acid and IMP taste in the T1r3 KO mice?

To address this question, we recorded L-amino acid and IMP-elicited calcium responses of TSCs from T1r3-GFP labeled mice and T1r3 KO mice to identify the involvement of different mGluRs in the detection of various L-amino acids and IMP. We used three L- amino acids: (1) monopotassium glutamate (MPG), (2) Ser, and (3) Arg - all of which are known to elicit different tastes in mice and humans, although CTA experiments with rats suggest these amino acids may also share some taste qualities (Schiffman et al., 1981; Delay et al., 2007; Eschle et al., 2009; Kawai et al., 2012). Additionally at high concentration, Ser elicits umami taste in humans (Kawai et al., 2012). We specifically studied the involvement of mGluR1 and mGluR4 in L-amino acid and IMP detection by examining the effects of selective mGluR1 and mGluR4 agonists on TSCs and the effect of selective antagonists on responses elicited by L-amino acids

and IMP. We found that some TSCs that respond to selective agonists also responded to L-amino acids and IMP. Additionally, the selective antagonists for both mGluR1 and mGluR4 significantly suppressed the responses elicited by L-amino acids and IMP. Our results suggest the involvement of both brain and taste variants of mGluR1 and mGluR4 in detection of IMP and L-amino acids.

3.3 Experimental Procedures

Animals used, most of the solutions, taste cells isolation procedure, and calcium imaging procedure used in this study is very similar to as described in Pal Choudhuri et al. (2015). The procedures are described in brief and any new information is described in detail.

3.3.1 Ethical Consideration:

All experimental procedures were reviewed and approved by the University of Vermont's Institutional Animal Care and Use Committee (IACUC protocol: 10-038). Mice were euthanized by CO_2 asphyxiation followed by cervical dislocation.

3.3.2 Animals:

Adult male and female T1r3-GFP (Damak et al., 2008), and T1r3 KO (Damak et al., 2003) mice were used in these experiments. Mice expressing enhanced green fluroscent protein (eGFP) under the control of the promoter of the *Tas1r3* gene were used primarily to confirm the isolation of TSCs. The T1r3 KO mice were generated on C57BL/6J background, and all 6 exons for the *Tas1r3* gene were eliminated. Breeding stock for T1r3-GFP and T1r3 KO mice were generously donated by Dr. Robert Margolskee (Damak et al., 2003, 2008). Expression of the GFP and the genetic deletion

of *Tas1r3* gene were verified by polymerase chain reaction (PCR) in all mice. All mice were maintained on a 12-h light/12-h dark cycle with food and water provided *ad libitum*.

For clarity, throughout the paper we are using T1r1 and T1r3 to refer to the receptor proteins in mice. In addition, terms such as umami, sweet and bitter are human perceptual constructs and are used primarily for effective communication.

3.3.3 Solutions and Chemicals:

Tyrode's solution contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, Glucose 10, and Na pyruvate 1. Ca^{2+}/Mg^{2+} free Tyrode's contained (in mM): NaCl 140, KCl 5, HEPES 10, Glucose 10, Na pyruvate 1, and EGTA 2. L-amino acids used as test solutions were (in mM): MPG 10, Ser 20, and Arg 10. MPG was used to ensure that responses were not due to the sodium component of MSG, as previously reported by other studies (Sako and Yamamoto, 1999; He et al., 2004). Di-sodium IMP was used at 1 mM. Physiologically relevant stimulus concentrations for each substance were chosen from behavioral and physiological data to ensure that each concentration was above recognition threshold in rodents (mice and rats) (Yoshii et al., 1986; Maruyama et al., 2006; Nakashima et al., 2012), but not high enough to cause any osmotic changes. The artificial sweetener, SC45647 (2-[[[[4-(aminomethyl)phenyl]amino]-[[(1*R*)-1-phenylethyl]amino]methyl]amino]ethane-1,1-diol) (100 μM) was used as a sweet stimulus (Nagarajan et al., 1996), and a mixture of cycloheximide (20 μM) and denatonium (2 mM) was used as a bitter stimulus. The L-amino acids were dissolved in Tyrode's solution and made fresh every day. Agonists and antagonists for glutamate receptors were purchased from Tocris (Bristol, UK). Agonists

used were: 30 µM – 100 µM (*S*)-3,5-Dihydroxyphenylglycine (DHPG) and 30 µM - 10 mM L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4). Antagonists used were: 10 µM – 40 µM 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), 50 µM – 100 µM D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5),100 µM (*RS*)-1-Aminoindan-1,5-dicarboxylic acid (AIDA), and 200 µM – 1 mM (*RS*)-α-Methylserine-*O*-phosphate (MSOP). DHPG is a group I mGluR (mGluR 1 and 5) agonist and L-AP4 is a selective group III mGluR (mGluR 4, 5, 7, and 8) agonist. CNQX is a potent AMPA/kinate antagonist. D-AP5 is a selective NMDA antagonist. AIDA is a selective group I mGluR antagonist and MSOP is a selective group III mGluR antagonist. Stock solutions of agonists and antagonists were prepared as recommended by the manufacturer and kept at -80^0C. DHPG and L-AP4 stock solutions were used within one week of preparation and antagonist stock solutions were used within 3 to 4 weeks of preparation. All test solutions were adjusted to approximately pH 7.4 using NaOH or HCl.

3.3.4 Taste Cell Isolation:

Taste cells from circumvallate and foliate taste buds were isolated using a protocol previously described by Pal Choudhuri et al. (2015), originally adapted from Behe et al. (1990) and Gilbertson et al. (1997). After euthanasia, tongues were removed and immersed in ice cold Tyrode's solution. An enzyme cocktail containing 0.8 mg/mL collagenase A (Sigma, St. Louis, MO), 1.5 mg/mL dispase II (Roche, Indianapolis, IN), 1 mg/mL trypsin inhibitor (Sigma, St. Louis, MO), and 0.05 mg/mL elastase (Worthington, Lakewood, NJ) was used to detach the lingual epithelium from the underlying mesenchyme. The tongue was then incubated in Tyrode's solution for 20 min followed

by incubation in Ca^{2+}/Mg^{2+}-free Tyrode's for another 20 min. In both solutions, oxygen was supplied continuously. The epithelium was gently removed from the underlying connective tissue and pinned flat with the epithelium surface down on a sylgard-lined petri dish. The tissue was incubated in the enzyme cocktail (without dispase II) for 5 min before being transferred to Ca^{2+}/Mg^{2+} free Tyrode's solution for 20-25 min. Taste buds and TSCs were removed from circumvallate and foliate papillae by gentle suction using fire polished glass micropipettes. TSCs were plated into a shallow recording chamber with a glass cover-slip pre-coated with Concanavalin A (Sigma, St. Louis, MO) to promote cell adherence. This protocol enabled us to reliably obtain both isolated taste cells and clusters of taste cells. Typically the cells were viable for 6-7 hours.

3.3.5 Calcium (Ca^{2+}) imaging:

Measurements of intracellular Ca^{2+} were obtained using the ratiometric fluorescent dye fura-2 AM (Molecular probes, Invitrogen Corporation, NY). Taste cells were incubated in 5-μM fura-2, AM and 0.05% pluronic F-127 dissolved in DMSO in Tyrode's solution for 25-30 min. The recording session began after bath washing the cells in Tyrode's solution for 10-20 min. Image capturing was done with an inverted fluorescent Nikon TE2000S microscope and C4742-95 digital camera. All solutions were bath applied using a gravity flow perfusion system. Test solutions were applied for 30 s before returning to Tyrode's solution. The antagonist CNQX was allowed to equilibrate with receptors for 10 min before application of agonist. D-AP5 was also applied either 10 min prior to agonist application or with the agonist. At the end of any test solution application, cells were washed in Tyrode's solution for 5 to 9 min. We performed a

desensitization study in which single cells were stimulated with the same L-amino acid for 4 to 5 times with varying wash times in between. We found that a wash of 5 to 9 min between stimulus applications was optimal for repeated responses of similar magnitude, although in some cells desensitization occurred independent of an extended wash. If the final stimulus application in the test sequence did not elicit a response, the cell was stimulated with a compound which previously elicited a response to make sure that the cell was still responsive. For the antagonist experiments, we excluded any cell from the analysis that did not recover during wash after antagonist application. Images were captured every 3 s during stimulus application and every 5 to 15 s during wash. In order to minimize cell damage during long wash periods, we limited image capturing during washes. After a response, images were captured only until the Ca^{2+} level went back to baseline (~2-3 min after the start of test solution application) and image capturing was resumed at least 1 min prior to next stimulus application. Fura2 AM was doubly excited at 340 nm and 380 nm and its emissions were recorded at 510 nm. Simple PCI 6.0 software (Hamamatsu, Sewickley, PA) running on a PC computer was used to capture images. Changes in Ca^{2+} concentrations are reported as F340/F380 plotted over time after background subtraction.

3.3.6 Quantification of Calcium Responses:

Increases in intracellular Ca^{2+}evoked by stimulus application were calculated as follows:

$$\Delta F = \frac{F_{Peak} - F_{Baseline}}{F_{Baseline}} * 100\%$$

where ΔF is the percent change above baseline, F_{Peak} is the largest F340/380 ratio within 1 min following the onset of a stimulus application, and $F_{Baseline}$ is the average of the five sampled F340/380 ratios immediately before stimulus application. Our criteria for defining ΔF as a response were: 1) the same stimulus elicited an increase in intracellular Ca^{2+} at least twice, and 2) each ΔF≥5%. Antagonist data were normalized using the average of Ca^{2+} responses to the stimulus applied immediately before and after the application of the mixture of the stimulus and antagonist.

3.3.7 Statistical Analysis:

Data are reported as means ± SEM. All statistical analyses were conducted with Graphpad Prism software. The statistical significance of differences between groups was calculated using paired or unpaired t-tests following Welch's correction, Wilcoxon matched pair signed rank test, and repeated measure ANOVA followed by Tukey's multiple comparison tests where applicable. An alpha of $P < 0.05$ was used to evaluate each analysis.

3.4 Results

Multiple receptors are known to be involved in the detection of umami or glutamate taste. These include T1r1+T1r3 heterodimer, mGluR4 (taste and brain variants), and mGluR1 (truncated and brain variants). While T1r1+T1r3 seems to be a generalist L-amino acid taste receptor, L-amino acids can elicit responses in TSCs of T1r1 KO and T1r3 KO mouse. This suggests other receptors are also capable of detecting L-amino acids (Damak et al., 2003; Maruyama et al., 2006; Kusuhara et al., 2013; Pal Choudhuri et al., 2015). The most likely candidates are the mGluRs already known to be

capable of detecting umami stimuli. To test this hypothesis, we used selective agonists and antagonists for mGluR1 and mGluR4 to determine if mGluRs underly calcium responses elicited by different L-amino acids. However, before we conducted these experiments, we needed to address potential issues related the impact of ionotropic glutamate receptors (iGluRs) on Ca^{2+} responses of TSCs.

In the taste bud, L-glutamate functions as a taste stimulus and potentially as a neurotransmitter. iGluRs are thought to be expressed on the basal end of TSCs where they may play a role in glutamate neurotransmission (Caicedo et al., 2000; Vandenbeuch et al., 2010). These receptors induce an increase in intracellular Ca^{2+} in the presence of micromolar concentrations of glutamate (Caicedo et al., 2000; Huang et al., 2012). In this study we used isolated TSCs and all stimuli were bath applied, thus some responses could be generated by iGluRs expressed in the basolateral membrane. Before the role of mGluRs could be explored, two important questions were addressed to avoid confounding our findings by iGluRs. The first was the potential role of iGluRs in generating responses in isolated TSCs and the second was to identify effective concentrations of iGluR antagonists. Since iGluRs can be activated by micromolar concentrations of glutamate, we first determined if a single TSC can respond to both micromolar (probable neurotransmission function) and/or millimolar (probable taste function) concentrations of glutamate. TSCs were stimulated with 50 µM and 10 mM MPG and the changes in intracellular Ca^{2+} levels were measured (Figure 3.1A, B). Five percent of the cells (19 of 389; Figure 3.1) responded (i.e. $\Delta F \geq 5\%$ in response to stimulation) to both concentrations of MPG. However, a greater number of cells

responded only to 10 mM glutamate (9%, 36 of 389) (Figure 3.1). The mean ΔF induced by 50 μM MPG (Mean±SEM=8.64±0.91, n=19) was significantly smaller than the ΔF induced by 10 mM MPG (Mean±SEM=42.84±9.48, n=36) (Figure 3.1B). Of the 55 cells that responded to 10 mM MPG, 34% (19 of 55) also responded to 50 μM MPG and 66% did not (Figure 3.1A). These data suggest that in some TSCs glutamate might have a neurotransmitter function, although they might also be taste responses (see discussion).

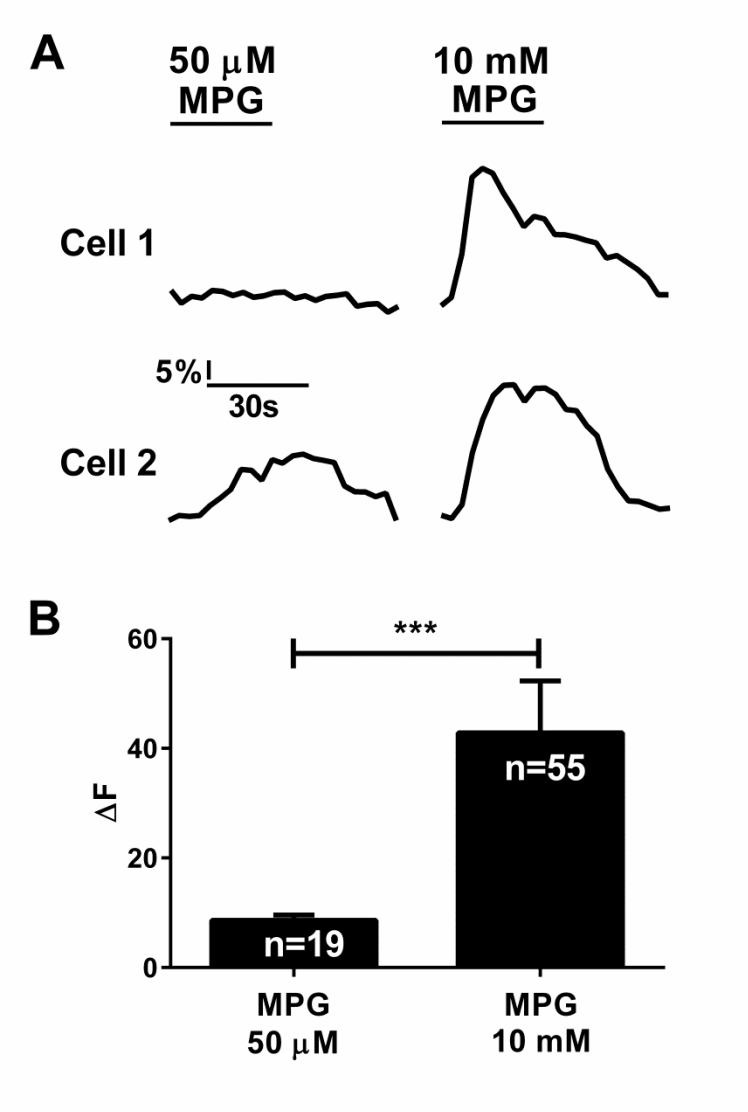

Figure 3.1: TSC responses to micromolar (50 µM) and millimolar (10 mM) concentrations of glutamate.
[A] Cell 1's Ca^{2+} responses are representative of TSCs that responded to millimolar but not micromolar concentrations of MPG. Cell 2's Ca^{2+} responses are representative of TSCs that responded to micromolar and millimolar concentrations of MPG. The bar above each stimulus trace represents the stimulus application period (30 sec). [B] Mean±SEM amplitude of Ca^{2+} increases above baseline for responsive cells only, i.e. cells with a $\Delta F \geq 5\%$. n = number of cells. *** $P < 0.001$.

To avoid NMDA- and AMPA/kinate-mediated responses in subsequent experiments, it was important to determine if a combination of NMDA (D-AP5) and AMPA/kinate (CNQX) receptor antagonists could be used effectively to block any contributions from iGluRs. Because we intended to use 10 mM MPG to investigate taste responses (see methods), we tested combinations of these selective antagonists mixed with 10 mM MPG. The effectiveness of three antagonistic mixtures were tested: (1) low (10 µM CNQX + 50 µM D-AP5), (2) medium (20 µM CNQX + 100 µM D-AP5), and (3) high (40 µM CNQX + 200 µM D-AP5). Even though the lowest concentration of each of the antagonists has been shown individually to completely block responses elicited by 300 µM glutamate in rat TSCs (Caicedo et al., 2000; Huang et al., 2012) , the low antagonists mixture did not have any effect on Ca^{2+} responses elicited by 10 mM MPG (n=7) in mice TSCs. However doubling those concentrations (medium antagonists mixture) reversibly and significantly suppressed responses to MPG (n=13 of 25 MPG responsive cells) (Figure 3.2). The high concentration antagonists mixture also significantly suppressed responses to MPG (n=7 of 12 TSCs responding to 10 mM MPG), but the effect was not reversible (Figure 3.2). Additionally the amount of antagonism by the high antagonists mixture was similar to that of the medium antagonists mix. Thus, for all subsequent experiments involving mGluR agonists and antagonists, we

used the medium antagonists mixture to block any iGluR-mediated responses whenever it was considered necessary.

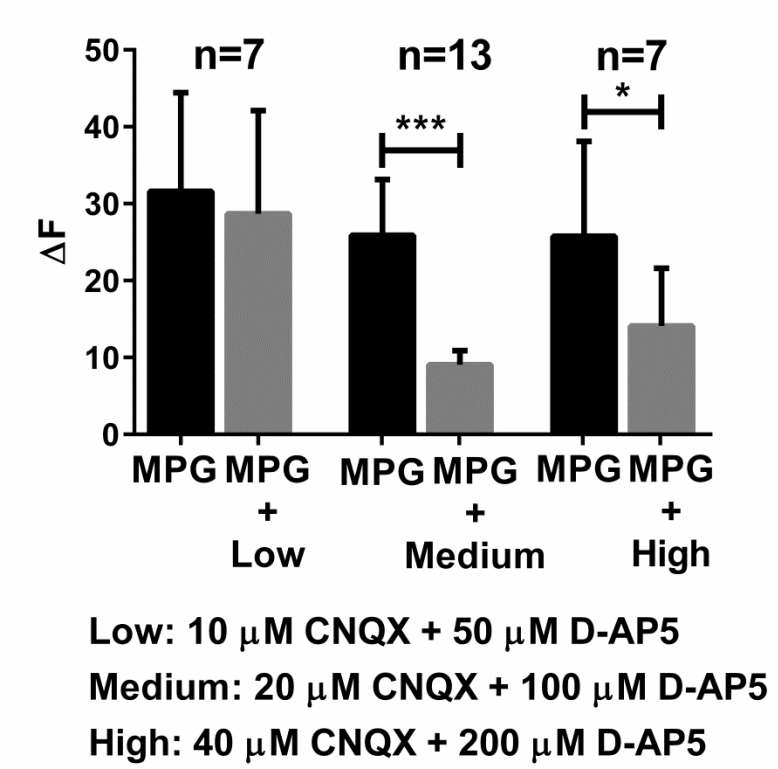

Figure 3.2: iGluR Antagonists mixtures partially block responses elicited by 10 mM MPG. Average effect of three different mixtures of CNQX+D-AP5 on Ca^{2+} responses elicited by 10 mM glutamate. Each antagonist mix was applied 10 min prior to and with MPG. Each bar represents the Mean±SEM amplitude of Ca^{2+} responses. n = number of cells. *** $P < 0.001$, * $P < 0.05$.

3.4. 1 mGluR1-mediates responses to L- amino acid and IMP:

mGluR1 is an excitatory receptor known to be expressed mainly in the postsynaptic somatodendritic region (Charpak et al., 1990; Staub et al., 1992; Crepel et al., 1994; Guerineau et al., 1995; Chavis et al., 1996) as well as perisynaptic regions (Nusser et al., 1994; Mateos et al., 2000) of neurons. A brain version as well as a truncated version of mGluR1 have been found to be expressed in the TSCs (Toyono et

al., 2003; San Garbiel et al., 2005, 2009) and are thought to elicit an umami taste (Kusuhara et al., 2013) in rodents. If mGluR1s contribute to the detection of L-amino acids and IMP, then a selective mGluR1 agonist would elicit Ca^{2+} responses in TSCs. In addition, selective antagonists for mGluR1 would block or suppress Ca^{2+} responses elicited by different L-amino acids and IMP.

mGluR1 Agonist

DHPG is a selective agonist for mGluR1 (Ito et al., 1992; Schoepp et al., 1994). In neuronal cells the EC_{50} of DHPG ranges between 10-30 µM depending on cell types (Conn and Pin, 1997; Lin et al., 1997). However, since concentrations above 100 µM can also activate other mGluRs, we tested DHPG at 30, 50 and 100 µM to determine an effective dose for subsequent testing (Conn and Pin, 1997; Lin et al., 1997). At 30 µM, DHPG did not elicit any responses in TSCs (n=180) (Figure 3.3A). At 50 µM, DHPG evoked responses in about 2% (n=3 of 180 cells) of the TSCs with a mean amplitude of Ca^{2+} responses of 7.28±1.16% (Mean ± SEM). At 100 µM, DHPG elicited Ca^{2+} responses in 12 TSCs (6%) with a mean ΔF amplitude of 10±1.3% (Mean ± SEM) (Figure 3.3A). Consequently, 100 µM DHPG was chosen for subsequent experiments.

Next we tested whether a single TSC would show a Ca^{2+} response to DHPG and an array of L-amino acids. Each TSC was stimulated with 5 different stimuli: (1) MPG (10 mM), (2) MPG (50 µM), (3) DHPG (100 µM), (4) Ser (20 mM), and (5) Arg (10 mM). At least one stimulus was capable of eliciting a Ca^{2+} response in 19 of 156 (12%) TSCs. Of the 19 responsive cells, 17 cells (89%) responded to 10 mM MPG and 6 cells (31%) responded to 50 µM MPG (Figure 3.3B). Since reports have shown that 10 µM of

CNQX and 50 µM of D-AP5 can completely block the Ca^{2+} responses elicited by 300 µM glutamate (Caicedo et al., 2000), responses elicited by 50 µM MPG in presence of CNQX and D-AP5 mixture are probably due to the activation of the brain version of mGluRs expressed in TSCs. In addition to MPG, TSCs also responded to DHPG (n= 6 of 19 cells; 31%), Ser (n=4 of 19 cells; 21%), and Arg (n=5 of 19 cells; 26%) (Figure 3.3B, Table 2.1). Because we were targeting TSCs responsive to MPG and DHPG, our data are biased towards MPG responsive cells. However, like our previous study, not every L-amino acid elicited a response in the same TSC (Pal Choudhuri et al., 2015). Of the 19 cells presented in Table 2.1, only 1 cell responded to all the stimuli. Additionally there were cells that responded to IMP and Arg, but not to MPG. The rest of the cells exhibited variable response patterns to the array of stimuli, similar to what was reported previously (Pal Choudhuri et al., 2015).

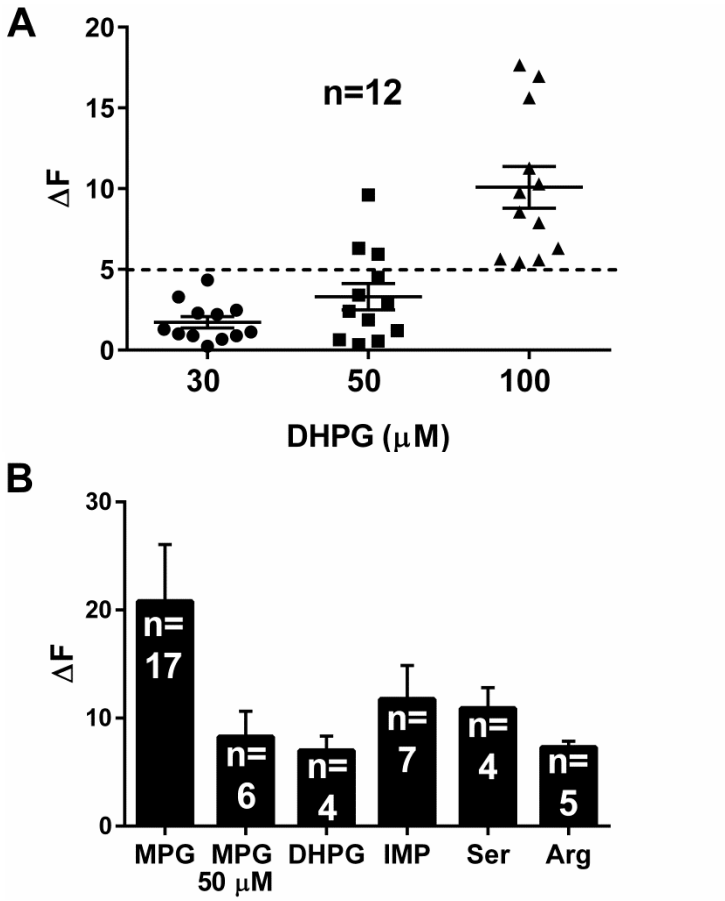

Figure 3.3: mGluR1 agonists (DHPG) and L-amino acids elicit responses in TSCs of T1r3-GFP mice. [A] TSC responses to 30, 50, and 100 μM DHPG (mGluR1 agonist). Dotted line represents the ΔF = 5% threshold, above which calcium levels are considered responses. None of the cells responded to 30 μM and all 12 cells responded to 100 μM DHPG. [B] Stimuli tested were DHPG (100 μM) and 3 L-amino acids with different side-chain groups: (MPG (10 mM and 50 μM), Ser (20 mM), and Arg (10 mM). Bars are Mean±SEM amplitudes of Ca^{2+} increases above baseline for responsive cells only, i.e. cells with a change in Ca^{2+} ≥5%. 17 of 19 cells responded to 10 mM MPG. Fewer TSCs responded to each of the other L-amino acids or DHPG and only 1 TSC responded to all stimuli. n = number of cells. **For these experiments, 20 μM CNQX was added to the bath solution, and 100 μM D-AP5 was added to each stimulus.**

Table3.1: Response patterns of 19 TSCs to IMP, L-amino acids, and mGluR1 agonist, DHPG.

TSC	MPG (10 mM)	MPG (50 µM)	DHPG (100 µM)	IMP (1 mM)	Ser (20 mM)	Arg (10 mM)
1	■	■	■	■	■	■
2	■	■	■	■		
3	■	■	■	■		
4	■	■		■		
5	■	■		■		
6	■	■				
7	■			■		■
8	■					■
9	■				■	■
10	■				■	
11	■				■	
12	■		■			
13	■					
14	■					
15	■					
16	■					
17	■					
18						■
19					■	

Each row represents an individual cell. Gray cells indicate Ca^{2+} responses to a stimulus solution (column). For these experiments, 20 µM CNQX was added to the bath solution, and 100 µM D-AP5 was added to each stimulus.

mGluR1 Antagonist

Next we recorded TSC responses to L-amino acids and IMP with the selective class I mGluR antagonist AIDA (Pellicciari et al., 1995; Moroni et al., 1997). AIDA blocks both mGlu1 and mGluR5. However, at this time there is no evidence that mGluR5 is expressed in TSCs. For all the L-amino acids and IMP, AIDA significantly and reversibly suppressed TSC responses (Figure 3.4A, B, C). The responses to 10 mM MPG, 20 mM Ser, and 1 mM IMP were reduced by 66% [n=29, $F(2,51)=78042$, $P < 0.0001$],

69% [n=12, $F(1,13)=27.57$, $P < 0.001$], and 65% [n=12, $F(1,14)=30.64$, $P < 0.0001$], respectively (Figure 3.4E, F, G). As a control, we also tested whether AIDA has an effect on a non-amino acid stimulus. Specifically, AIDA did not had any effect on Ca^{2+} responses (n=5) elicited by the potent artificial sweetener, SC45647 (Figure 3.4D). Additionally, 100 μM AIDA alone did not elicit any responses in TSCs (n=5).

Since previous studies have shown that mGluR selective agonists can elicit responses in cells expressing T1r1+T1r3, we assumed that it is also possible that selective mGluR antagonists might have an effect on T1r1+T1r3 and limited testing of mGluR1 antagonists to TSCs of T1r3 KO mice. Similar to T1r3-GFP mice, in T1r3 KO cells AIDA also significantly and reversibly suppressed responses elicited by MPG [n=9, $F(2,16)=15.25$, $P < 0.001$], Ser [n=6, $F(2,8)=20.13$, $P < 0.001$), Arg [n=19, $F(2,28)=58.35$, $P < 0.0001$], and IMP [n=9, $F(2,14)=35.22$, $P < 0.0001$] (Figure 3.5 A, B, C, D, E, F, G, H). These data suggest that at least in the posterior tongue, L-amino acids and IMP can also be detected by mGluR1.

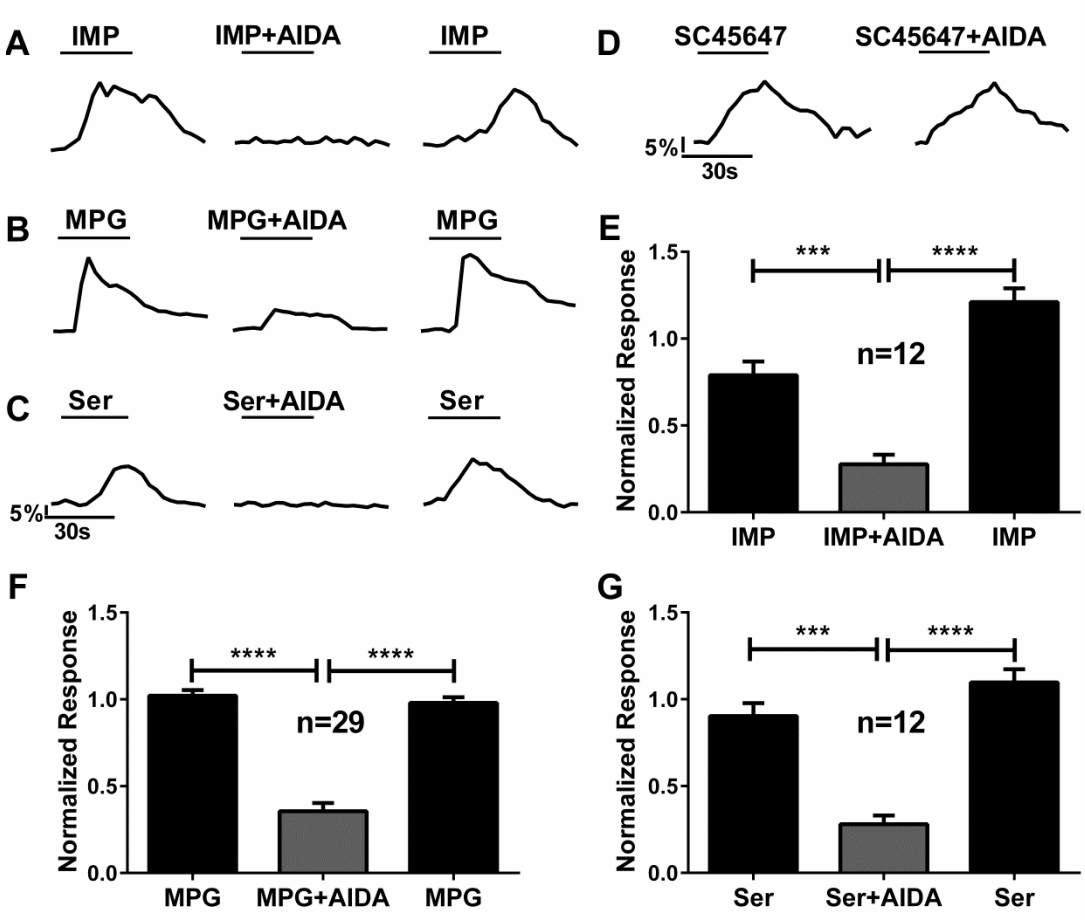

Figure 3.4: Effect of 100 µM AIDA (group I mGluR selective antagonist) on L-amino acids and IMP elicited responses in TSCs of T1r3-GFP mice.
The antagonist was tested against MPG (10 mM), IMP (1 mM), and Ser (20 mM). [A, B, C] In TSCs, AIDA partially or completely inhibited responses to IMP, MPG, and Ser, respectively. Traces are representative of responses during pretest stimulation, stimulation with AIDA, and posttest stimulation [D] AIDA had not effect on SC45647 elicited response [E, F,G] Average effect of AIDA on all responses to IMP, MPG, and Ser, respectively. Each bar represents Mean±SEM amplitude of Ca^{2+} responses during pretest, test and posttest stimulation. n = number of cells. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$.

3.4.2 mGluR4-mediates responses to L- amino acid and IMP:

mGluR4 receptors belong to the group III mGluR family and are predominantly expressed presynaptically in the CNS (Bradley et al., 1999; Corti et al., 2002; Niswender and Conn, 2010). A brain variant as well as a taste variant of mGluR4 are found in the TSCs and appear to contribute to umami taste (Chaudhuri et al, 1996; Delay et al., 2004;

Nakashima et al. 2001; Toyono et al., 2002). If mGluR4 contributes to the detection of L-amino acids and IMP, then a selective mGlu4 agonist such as L-AP4 would elicit Ca^{2+} responses in TSCs and a selective antagonist for mGlu4 such as MSOP would block the responses elicited by different L-amino acids and IMP. However, L-AP4 can also elicit responses in cells expressing the umami receptor T1r1+T1r3 heterodimer (Nelson et al., 2002). To eliminate any influence of the T1r1+T1r3 heterodimer, we performed all of these experiments using TSCs of T1r3 KO mice.

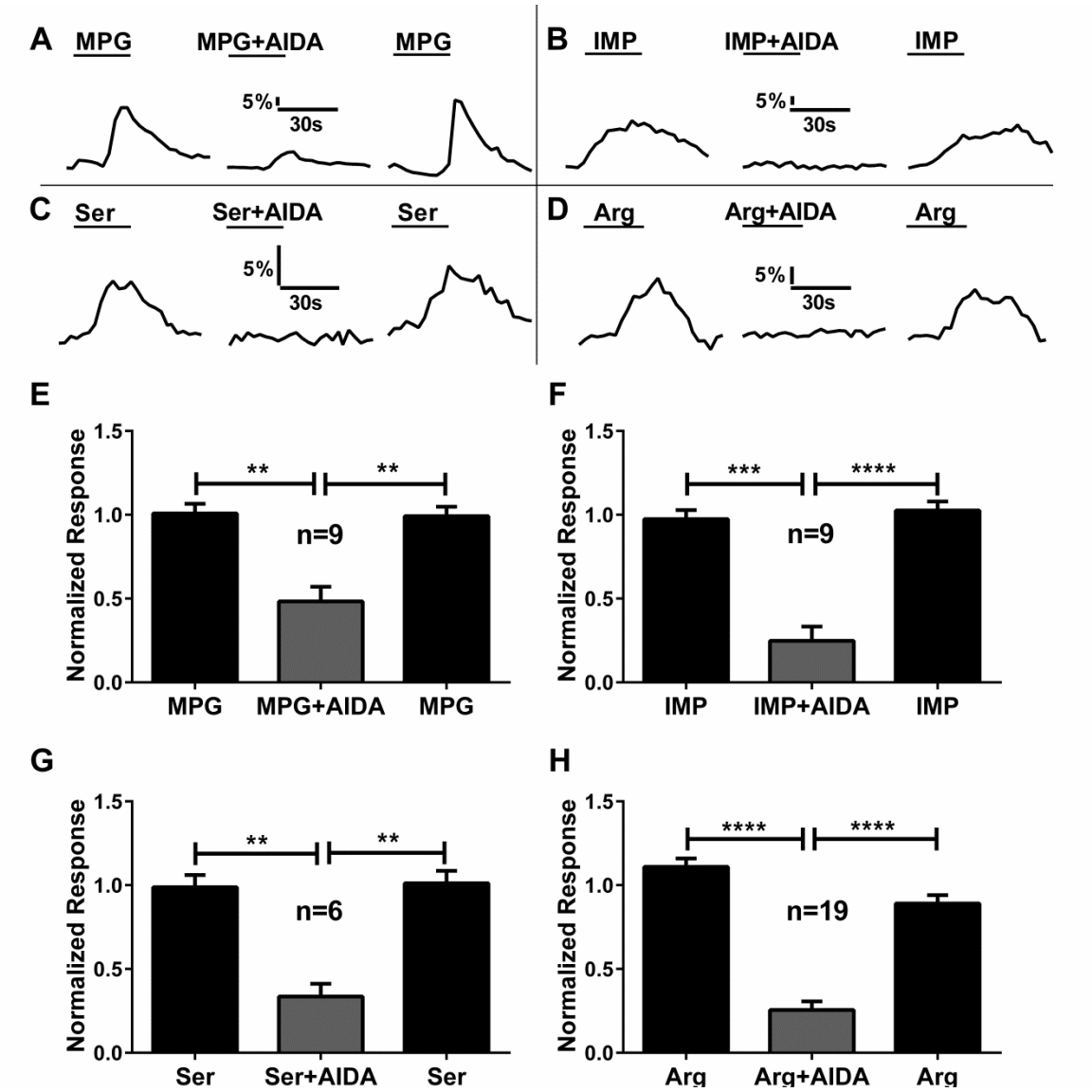

Figure 3.5: Effect of 100 µM AIDA (group I mGluR selective antagonist) on Ca^{2+} responses elicited by L-amino acids and IMP in TSCs of T1r3 KO mice.
The antagonist was tested against MPG (10 mM), IMP (1 mM), Ser (20 mM), and Arg (10 mM). **For these experiments, 20 µM CNQX was added to the bath solution, and 100 µM D-AP5 was added to each stimulus.** [A, B, C, D] In TSCs, AIDA partially or completely inhibited responses to MPG, IMP, Ser, and Arg, respectively. Representative traces of Ca^{2+} responses during pretest, test, and posttest stimulation for each L-amino acid and IMP. [E, F, G, H] Average effect of AIDA on all Ca^{2+} responses to MPG, IMP, Ser, and Arg, respectively. Each bar represents the Mean±SEM amplitude of Ca^{2+} responses. n = number of cells. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$.

mGluR4 Agonist

L-AP4, a glutamate analog, is a selective agonist for group III mGluRs (Nakanishi, 1992; Bushell et al., 1995; Tones et al., 1995), but of that group, only mGluR4s have been detected in the TSCs. Behavioral studies in rodents (Chaudhuri et al 1996; Delay et al., 2000, 2004; Nakashima et al., 2001, 2012) suggest that L-AP4 elicits a taste similar to glutamate. We first tested whether L-AP4 can elicit Ca^{2+} responses in TSCs of T1r3 KO mice. We determined the concentration-response relationship for L-AP4 with 0.05, 0.1, 0.5, 1, 3, 5, and 10 mM and found the EC_{50} for L-AP4 to be about 3.7 mM (Figure 3.6). Therefore, in subsequent experiments we used 4 mM of L-AP4.

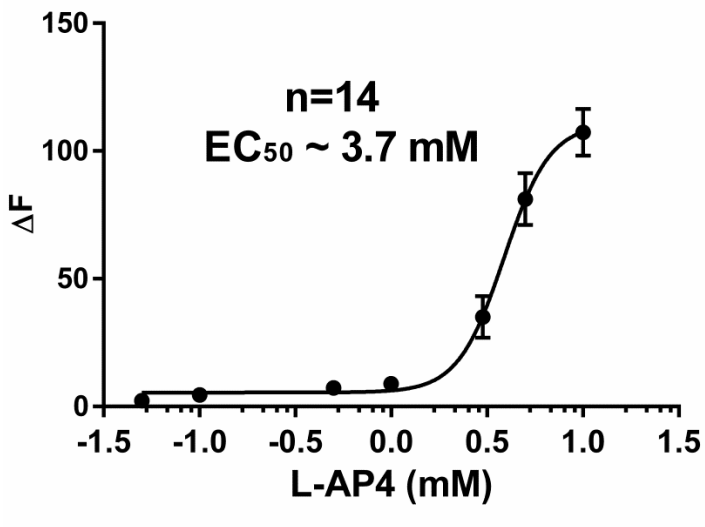

Figure 3.6: L-AP4 (group III mGluR selective agonist) elicited responses in TSCs of T1r3 KO mice in a concentration-dependent manner.
Concentration-response relationship for L-AP4. Each point represents the Mean±SEM Ca^{2+} response for that concentration. X-axis represents log values of L-AP4 concentrations. Increases in L-AP4 concentration elicited dose-dependent increases in Ca^{2+} responses. EC_{50} was calculated to be about 3.7 mM.

Next we determined whether a single TSCs would produce a Ca^{2+} response to an array of L-amino acids and L-AP4. Each TSC was exposed to 5 different stimuli: (1)

MPG (10 mM), (2) Ser (20 mM), (3) Arg (10mM), (4) IMP (1mM) and (5) L-AP4 (4 mM). At least one stimulus was capable of eliciting a Ca^{2+} response in 6 of 59 (10%) TSCs. Interestingly, some L-AP4-responsive cells also responded to one or more of the L-amino acids and IMP. Of the 6 responsive TSCs, 4 cells (66%) responded to L-AP4. Along the same line 4, 4, and 3 of the 6 cells responded to MPG, Ser, and Arg, respectively, and 1 cell responded to IMP.

mGluR4 Antagonist

Even though the mGluR4 antagonist, CPPG, has been shown to block glutamate responses in behavioral, nerve recording, and Ca^{2+} imaging studies and is highly potent for mGluR group III, it can also antagonize other mGluRs at higher concentrations (Jane et al., 1996; Toms et al., 1996). On the other hand MSOP is a selective group III mGluR antagonist with no known effect on postsynaptic mGluRs or iGluRs (Thomas et al., 1996). Thus we used MSOP to study the involvement of mGluR4 in detection of L-amino acids and IMP.

MSOP significantly suppressed TSC responses to 4 mM L-AP4 in a dose dependent manner [n=5; $F(1,5)=13.37$, $P < 0.05$] (Figure 3.7A, B). At 500 μM MSOP significantly reduced L-AP4 responses. Since higher concentrations did not suppress the response any further, 500 μM MSOP was used in subsequent experiments. As a control, MSOP was tested to determine if it has any effect on Ca^{2+} responses elicited by a stimulus of another taste quality. MSOP did not had any effect on SC45647-elicited Ca^{2+} responses (n=4) nor did it have any effect on TSCs when presented alone at concentrations up to 1 mM (n=5).

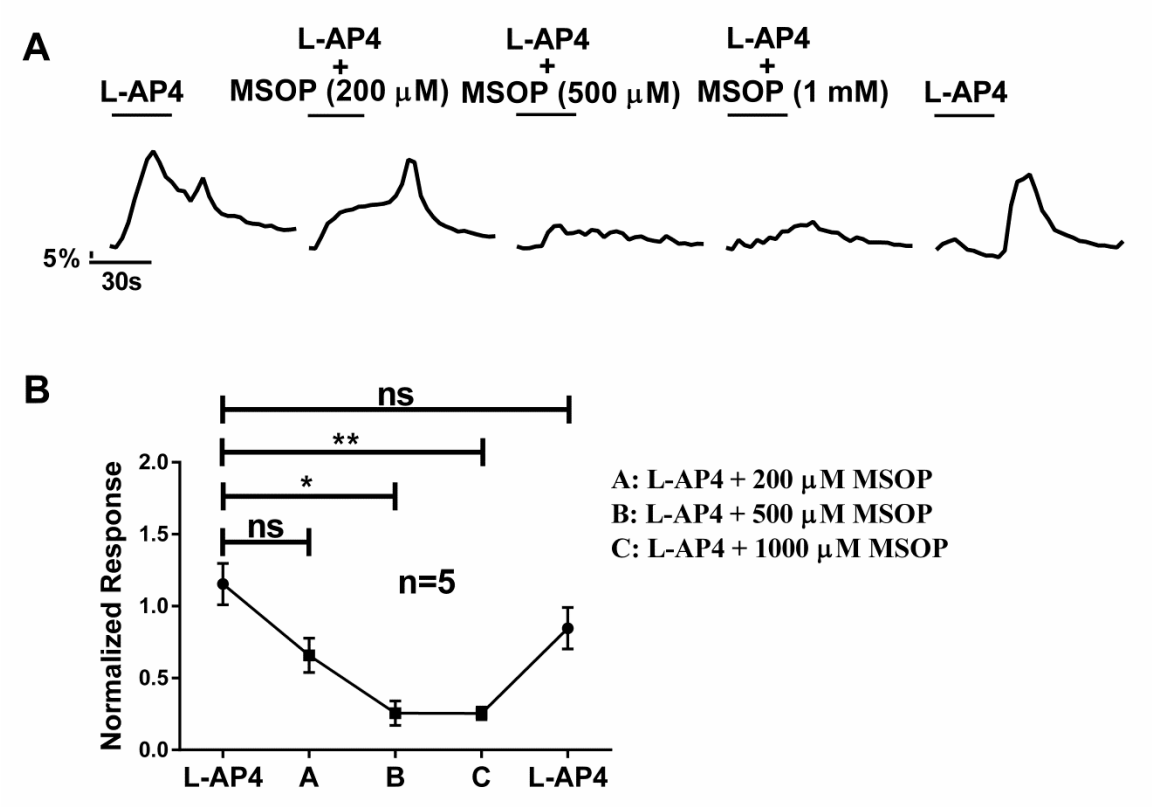

Figure 3.7: MSOP (group III mGluR selective antagonist) partially inhibited L-AP4 (4 mM) mediated responses of TSCs of T1r3 KO mice in a concentration-dependent manner.
[A] Representative Ca^{2+} responses in a single cell showing inhibition of 4 mM L-AP4-elicited responses by MSOP in a concentration-dependent manner. L-AP4 was presented alone before and after the three trials with L-AP4+MSOP. [B] Average effect of MSOP on L-AP4 mediated responses. Each point represents Mean±SEM response for each stimulus solution. **For these experiments, 20 μM CNQX was added to the bath solution, and 100 μM D-AP5 was added to each stimulus.** n = number of cells. ** $P < 0.01$, * $P < 0.05$. ns = no significant difference.

MSOP significantly and reversibly suppressed responses evoked by the L-amino acids and IMP (Figure 3.8). MSOP suppressed responses to 10 mM MPG, 20 mM Ser, 10 mM Arg, and 1 mM IMP by 54% [n=9, $F(1,11)=5.73$, $P < 0.05$], 63% [n=6, $F(1,7)=5.29$, $P <0.05$], 56% [n=12, $F(1,5)=11.15$, $P < 0.01$], and 61% [n=20, $F(1,25)=31.14$, $P < 0.0001$], respectively (Figure 3.8A, B, C, D, E, F, G, H). These data suggest that at least in the posterior tongue, mGluR4 acts as an L-amino acid and IMP receptor.

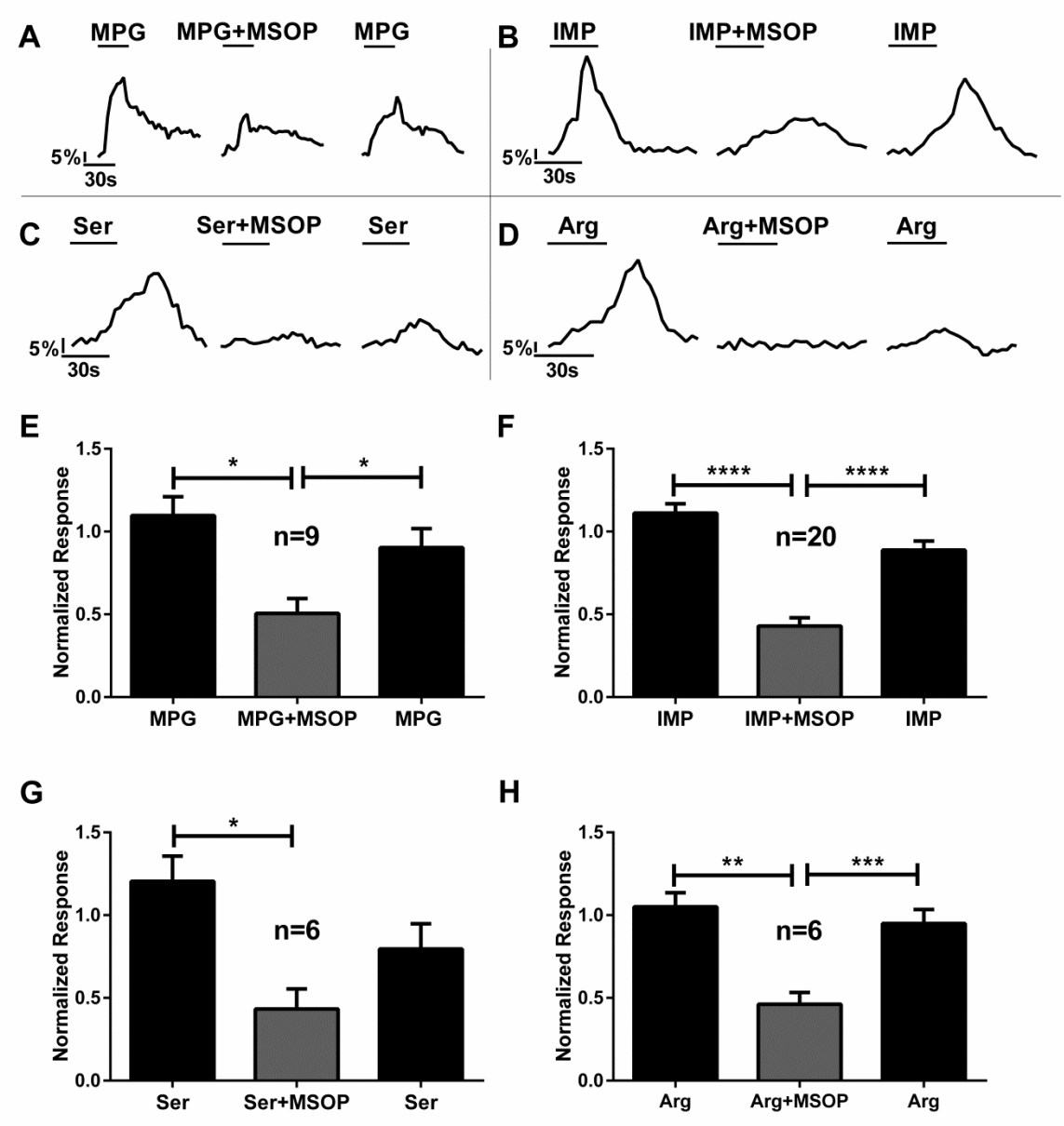

Figure 3.8: Effect of 500 µM MSOP (group III mGluR selective antagonist) on L-amino acids- and IMP-elicited responses in TSCs of T1r3 KO mice.
The antagonist was tested against MPG (10 mM), IMP (1 mM), Ser (20 mM), and Arg (10 mM). For these experiments, 20 µM CNQX was added to the bath solution, and 100 µM D-AP5 was added to each stimulus. [A, B, C, D] In TSCs, MSOP partially or completely inhibited responses to MPG, IMP, Ser, and Arg, respectively. In TSCs, MSOP partially or completely inhibited responses to MPG, IMP, Ser, and Arg, respectively. [E, F, G, H] Average effect of MSOP on all responses to MPG, IMP, Ser, and Arg, respectively. Each column represents Mean±SEM amplitude of Ca^{2+} responses during pretest, test and posttest stimulus conditions. n = number of cells. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$.

3.5 Discussion

Detection of L-amino acids has been tied to taste receptors associated with umami taste, especially the T1r1+T1r3 heterodimer (Nelson et al., 2002; Li et al., 2002). However, there is a growing body of evidence suggesting additional glutamate receptors, such as the mGluRs, may be involved in detecting umami and that at least some of these receptors are likely to participate in the taste of other L-amino acids. Although T1r1+T1r3 has been proposed to be a broadly tuned L-amino acid receptor, we have previously shown that glutamate-responsive TSCs of wild type mice respond to some amino acids but generally not to all, and that L-amino acid-responsive TSCs may respond to multiple L-amino acids but not necessarily to glutamate (Pal Choudhuri et al., 2015). We also found that L-amino acids can elicit responses in TSCs of T1r3 KO mice. An IMP binding site located in the N-terminal domain of the T1r1 subunit has been proposed (Zhang et al., 2008), but the lack of IMP-induced Ca^{2+} responses in HEK cells expressing T1r1 or the heterodimer T1r1+T1r3 (Nelson et al., 2002) raises questions about its role as an independent IMP taste receptor. Thus we investigated whether mGluRs, specifically mGluR1 and mGluR4, can function as L-amino acid and IMP taste receptors. The principal finding in this study is that these mGluRs are involved in the detection of L-amino acids and IMP. The pharmacological characterization of the responses elicited by L-amino acids and IMP indicate that both mGluR1 and mGluR4 can detect L-amino acids and IMP in TSCs, and are probably the main candidate receptors underlying the Ca^{2+} responses in TSCs of T1r3 KO mice to these stimuli.

The metabotropic glutamate receptors mGluR1 and mGluR4 were originally characterized from brain tissue. In situ hybridization, immunoblot, and immunohistochemical analysis have shown that these receptors are also expressed in taste buds (Chaudhari et al., 1996; Yang et al., 1999; Chaudhari et al., 2000; San Gabriel et al., 2005, 2009a). For both receptor types, in addition to the brain version, a structurally and functionally distinct form was also found in the taste buds and were termed truncated or taste variants of mGluR1 and mGluR4. The taste-mGluR1 lacks most of the N-terminus VFT domain. Similarly, the taste-mGluR4 also lacks 50% of the receptor's extracellular N-terminus domain. Since the N-terminus VFT domain is considered to be essential for L-glutamate binding, theses truncations are likely to influence the affinity of the receptor for glutamate. For both receptors the affinity of the receptor in vitro expression system was lower for the truncated variant than for the brain variant (Chaudhari et al., 2000; San Gabriel et al., 2009a). While brain-expressed variants of the receptors can be activated by micromolar concentrations of glutamate, the truncated receptors responded only at concentrations ≥1 mM of glutamate. This reduced sensitivity of the truncated-mGluRs is of physiologic significance as in many behavioral and cellular studies, this corresponds well with the concentrations of agonists needed to elicit taste responses and suggests that the truncated versions of the mGluRs mainly function as taste receptors (Chaudhari et al., 2000; San Gabriel et al., 2009a) . Behavioral and cellular studies have shown that micromolar concentrations of glutamate can also elicit significant behavioral responses, suggesting that brain mGluRs also function as taste receptors (Nakashima et al., 2012). Since iGluRs have been reported in tongue epithelium and iGluRs can also be activated by micromolar concentrations of glutamate, we first

aimed to characterize and block any iGluR mediated responses to specifically study mGluR activity.

During the early 1990's glutamate or umami taste was hypothesized to involve iGluRs, such as NMDA and non-NMDA type receptors expressed in TSCs (Lin and Kinnamon, 1999; Brand 2000). Later, studies showed that while rats and mice could taste NMDA, their behavior indicated that by itself, NMDA did not elicit a taste similar to glutamate (Chaudhuri et al., 1996; Stapleton et al., 1999; Nakashima et al., 2001; Delay et al., 2004). In addition to being implicated in umami taste, glutamate was also proposed to function as a neurotransmitter activating iGluRs in the taste system (Caideo et al., 2000; Vandenbeuch et al., 2010; Niki et al., 2011; Huang et al., 2012). To date there is no direct evidence that establishes a neurotransmitter-like function for glutamate for TSCs in the taste buds. There is evidence, however, that glutamate may act as an efferent transmitter in the taste bud. This is based mainly upon the expression of glutamate aspartate transporter in type I TSCs and the expression of vesicular transporters for glutamate, VGLUT 1 and 2, in the nerve fibers surrounding taste buds (Vandenbeuch et al., 2010; Huang et al., 2012). These studies suggest that glutamate may be released by afferent nerve fibers on TSCs, and may modulate taste function. Thus iGluRs may have multiple afferent taste functions, depending upon whether they are expressed in the membrane at the apical end of the TSC (taste) or along the basolateral portion of the membrane (synaptic function).

Since in our study all stimuli were bath-applied, it is possible some responses may be due to activation of iGluRs expressed throughout the plasma membrane of TSCs. In

the central nervous system (CNS), micromolar concentrations of glutamate can activate iGluRs and, in the taste system, 30 µM glutamate can elicit iGluR-mediated responses in TSCs (Caicedo et al., 2000). To block iGluR responses associated with neural modulation/transmission, 20 µM CNQX combined with 100 µM D-AP5 were added to all experimental solutions. These are well above concentrations that effectively block NMDA and AMPA receptors within the CNS and should be similarly effective at blocking iGluRs that might be involved in synapse-associated glutamate activity, enabling us to selectively study the involvement of mGluRs in the detection of L-amino acids and IMP. The iGluR antagonist mixture significantly reduced Ca^{2+} responses elicited by 10 mM MPG in approximately half of the TSCs tested (Figure 3.2). This finding suggests an iGluR-mediated function in taste buds. Further study would be required to determine if iGluRs might function as L-amino acid taste receptors.

Even in the presence of iGluR antagonists, 35% of the 10 mM MPG responsive cells also responded to 50 µM MPG, suggesting activation of brain variant mGluRs in TSCs. Threshold experiments reported by Nakashima et al. (2012) have shown that mice can detect glutamate and the selective group III mGluR agonist L-AP4, even at micromolar concentrations, albeit weakly compared to their responds when these substances are presented at millimolar concentrations. CTA studies against L-AP4 and glutamate also gave comparable results (Nakashima et al., 2001; Delay et al., 2002). Moreover, group III and group I glutamate receptor antagonists, CPPG and AIDA were able to block CTAs elicited by micromolar glutamate and L-AP4 (Eschle et al., 2009 Nakashima e al., 2012). Based on these behavioral studies, we can infer that calcium

responses elicited by micromolar concentrations of MPG in our study are taste responses mediated by multiple candidate receptors, including: (1) brain expressed variant of mGluR1, (2) brain expressed variant of mGluR4, (3) other brain expressed variants of mGluRs, and/or (4) mGluR heterodimers.

3.5.1 mGluR1 detects L-amino acids and IMP in mouse TSCs

We used selective agonists and antagonists to investigate the role of group I mGluRs in detection of L-amino acids and IMP. For an mGluR1 agonist, we used DHPG, an orthosteric agonist for group I mGluRs with similar potencies at mGluR1 and mGluR5 (Brebet et al., 1995; Wisniewski and Car, 2002). At low concentrations, DHPG is highly selective for group I mGluRs. However, it's EC_{50} for group II mGluR is 106 µM and for group III mGluR is > 1000 µM (Brebet et al., 1995; Conn and Pin, 1997; Wisniewski and Car, 2002). Although to date there is no evidence that suggests a role for group II mGluRs in umami or L-amino acid detection, Toyono et al. (2007) reported the expression of mGluR2/3 in taste epithelium. Thus we limited the highest concentration of DHPG to 100 µM. Because the potency of DHPG is similar to that of glutamate (Wisniewski and Car, 2002), we did not expect to see many responses with low concentrations of DHPG. As expected, only 1% and 6% of the TSCs responded to 50 µM and 100 µM of DHPG, respectively. Since the truncated taste-mGluR1 is likely to be activated by millimolar concentrations of glutamate, DHPG-activated Ca^{2+} responses are probably mediated by the brain-mGluR1 variant.

Some TSCs that responded to DHPG also responded to Ser and Arg, indicating that these and possibly other L-amino acids also activate mGluR1. All cells that

responded to 100 µM DHPG also responded to 50 µM MPG and vice versa. But all cells that responded to 10 mM MPG did not necessarily respond to 100 µM DHPG (Table 3.1). This was expected since the potency of DHPG is similar to that of glutamate. In a HEK cell expression system, the T1r1+T1r3 receptor was activated by glutamate (50 mM) only in the presence of IMP, whereas some L-amino acids such as Ser and Arg alone were able to evoke Ca^{2+} responses in those HEK cells (Nelson et al., 2002). Thus, 10 mM MPG elicited responses were probably mediated by the activation of truncated taste-mGluRs with a lower affinity for glutamate compared to the brain-expressed versions of mGluRs. Additionally, some cells that responded to L-amino acids did not respond to MPG or DHPG, suggesting the activation of T1r1+T1r3 or calcium sensors in those instances.

The antagonist data for mGluR1 further strengthened the possibility of a role of mGluR1 in L-amino acid and IMP detection. AIDA, a selective antagonist of mGluR1, AIDA, significantly and reversibly suppressed responses elicited by MPG, Ser, Arg and IMP in TSCs of T1r3-GFP as well as T1r3 KO mice (Figure 3.4, 3.5). AIDA has been shown to have no effect on group II (mGluR2) or group III (mGluR4) receptors expressed individually in baby hamster kidney cells. Additionally, it has no effect on ionotropic glutamate receptors (Pellicciari et al., 1995; Moroni et al., 1997). This suggests that L-amino acids and IMP can activate mGluR1 in TSCs. Although to date, there is no evidence for the expression or activation of mGluR5 on TSCs, we cannot completely rule out that possibility. Still, when considered together, our data strongly

suggest that L-amino acids and IMP can evoke responses in TSCs by activation of mGluR1.

3.5.2 L-amino acids and IMP can activate mGluR4 in mouse TSCs

Several behavioral, Ca^{2+} imaging, and nerve recording studies have indicated a role for mGluR4 in the detection of umami taste (Chaudhari et al., 1996, 2000; Delay et al., 2000, 2004; Kusuhara et al., 2013). Brain and taste variants of mGluR4 have been shown to be expressed in the posterior portion of the taste epithelium. Evidence for the function of mGluR4 as a taste receptor comes from the fact that the selective mGluR4 agonist, L-AP4, physiologically and behaviorally mimic the response of glutamate (Chaudhari et al., 1996, 2000; Delay et al., 2000; Nakashima et al., 2001; Delay et al., 2004; Nakashima et al., 2012; Kusuhara et al., 2013). Additionally, mGluR4 antagonists CPPG and MPPG block physiological and behavioral responses evoked by glutamate (Eschle et al., 2009; Yasumatsu et al., 2012; Kusuhara et al., 2013). Although L-AP4 is selective for group III mGluRs, it has been shown to activate the T1r1+T1r3 heterodimer expressed in HEK cells in presence of IMP (Nelson et al., 2002). Similar evidence that L-AP4 is able to activate these receptors in vivo have not yet been reported. Even so, responses elicited by L-AP4 in wild type mice may in part result from activation of the T1r1+T1r3 heterodimer, thus may not clearly verify the role of mGluR4 in taste function. In this study a micromolar concentration (100 μM) of L-AP4 could elicit Ca^{2+} responses in TSCs of T1r3 KO mice. However, the dose-response analysis with L-AP4 revealed a half-maximal effect (EC_{50}) of 3.7 mM (Figure 3.6). These data indicate that L-AP4 activated the brain as well as the truncated variants of mGluR4 in this study. We further

showed that L-AP4 responses can be suppressed by the selective mGluR4 antagonist MSOP.

Interestingly, most of the L-AP4 responsive cells also responded to L-amino acids and IMP and those responses were also significantly suppressed by MSOP, strongly suggesting a role for mGluR4s in L-amino acid and IMP taste responses (Figures 3.7, 3.8). Most of the previous studies used CPPG as the mGluR4 antagonist. Although CPPG is a very potent antagonist at mGluR4, it can also effect other mGluRs. Thus we used MSOP which is less potent than CPPG but is highly selective for mGluR4. Additionally it also does not have any known effect on iGluRs. Consequently, our data provide strong evidence for the activation of mGluR4 by L-amino acids and IMP in TSCs.

From our data it is not possible to identify whether mGluR1 and mGluR4 are expressed in the apical or the basolateral membrane of TSCs. In the central nervous system, mGluR1 is generally expressed on the postsynaptic cells (Charpak et al., 1990; Staub et al., 1992; Crepel et al., 1994; Nusser et al., 1994; Guerineau et al., 1995; Chavis et al., 1996; Mateos et al., 2000) and mGluR4 is predominantly expressed presynaptically (Bradley et al., 1999; Corti et al., 2002; Niswender and Conn, 2010). If glutamate has a neurotransmitter/modulatory function in taste cells and is released by nerve fibers to work on the TSCs, then it's function might be mediated by mGluR1 or mGluR4 expressed on the basolateral membrane of the TSCs. However immunoblot and immunohistochemical analyses have shown that both variants of mGluR1 taste-mGluR4 are present in the taste pores, and no immunostaining for mGluR4 was found in taste cells below taste pore in synaptic regions (Toyono et al., 2002, 2003). Additionally, several

behavioral and physiological studies have strongly suggested the activation of brain as well as truncated variants of mGluR1 and 4 in glutamate taste detection (Chaudhari et al., 2000; Delay et al., 2002; Eschle et al., 2009; Nakashima et al., 2012; Yasumatsu et al., 2012; Kusuhara et al., 2013). The data reported in this study are in agreement with these previous studies and, taken as a whole, these findings argue that both variants of both mGluR1 and mGluR4 are involved in the taste of L-amino acids and IMP.

Neither mGluR1 nor mGluR4 antagonist was able to completely block the responses elicited by L-amino acids or IMP. From our data, it is not possible to determine if different receptors, i.e., an mGluR1 and T1r1, can generate responses in the same T1r3 KO cell, or if there might be other receptors that act as candidate receptors for IMP and L-amino acids taste in these cells. mGluR2 and mGluR3 are also expressed in the tongue, however, their expression in the taste cells is not yet established. Even so, they may also be potential candidate receptors in T1r3 KO TSCs. Recently, the Ca^{2+} sensor CaSR and the class C GPCR, GPRC6A, which can also detect L-amino acids, were localized to Type I and III taste cells (San Gabriel et al., 2009b; Bystrova et al., 2010). One very intriguing possibility is that taste-mGluR4 and/or some other receptors may form a complex with each other, or with T1rs that responds to L-amino acids and IMP. However, further investigation is needed to examine this hypothesis.

A synergistic response between an umami substance and a 5'-ribonucleodite such as IMP is a characteristic property of umami taste (Delay et al., 2000). We have previously showed that, like MPG, other L-amino acids can also elicit synergistic responses in TSCs when mixed with IMP (Pal Choudhuri et al., 2015). Although the

intracellular signaling pathway for synergistic response is not yet understood, one hypothesis is the downregulation of cAMP pathway which in turn causes the downregulation of PKA and subsequent release of phosphorylation and further activation of PLC mediated pathway (Kinnamon and Vandenbeuch, 2009). Since activation of mGluR4 causes downregulation of the cAMP pathway, it might be involved in synergistic responses either by itself or in combination with other receptors. Additionally we have previously demonstrated the occurrence of synergistic responses in the TSCs of T1r3 KO mice. Thus, mGluRs individually, dimerized with T1r1, or possibly as a component of some form of receptor complex might also be involved in the synergistic responses. Additional research is needed to determine the role of mGluRs, if any, in generating synergistic responses.

3.5 Conclusions

In summary we have shown that isolated mouse TSCs can respond to a single L-amino acid and IMP, and to selective agonists of mGluR1 or mGluR4 receptors. Additionally selective antagonists for mGluR1 and mGluR4 suppressed responses elicited by different L-amino acids and IMP. Our data strongly suggest the involvement of mGluR1 and mGluR4 in L-amino acid and IMP taste sensations and provides an explanation for residual L-amino acid and IMP responses in T1r3 KO mice.

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Chapter 4 : CONCLUSIONS AND FUTURE DIRECTIONS

Umami taste has high potential for increasing the palatability of food, and can be effectively used to fight against malnutrition in elderly and people with dietary challenges (Schiffman, 2000; Yamaguchi & Ninomiya, 2000; Bellisle, 2008; Toyama, Tomoe, Inoue, Sanbe, & Yamamoto, 2008; Yamamoto, Tomoe, Toyama, Kawai, & Uneyama, 2009). The exemplar L-amino acid that elicits umami taste is L-glutamate. It is readily available in many foods and the amount of free glutamate increases with the processing or aging of the food. For over a decade the receptor system and transduction mechanisms for L-glutamate taste have been heavily investigated. While many mysteries have been solved, new questions have unfolded, and some questions are still remaining to be answered. One open question involves the identification of additional L-amino acid taste receptors.

Along with glutamate, other L-amino acids are also present in food and elicits sweet, bitter, umami or a combination of those taste qualities (Kawai, Sekine-Hayakawa, Okiyama, & Ninomiya, 2012). However, research focused on L-amino acid taste has not been as intense as it was for L-glutamate. Two other important players of umami taste are the 5' ribonucleotides, inosine 5' monophosphate (IMP) and guanosine 5' monophosphate (GMP). Along with eliciting an umami taste, these nucleotides can also synergistically potentiate the taste of glutamate, thus participate in making food even more flavorful (Bellisle et al., 1991; Lin & Kinnamon, 1998; Yamaguchhi & Ninomiya, 2000; Lin, Ogura, & Kinnamon, 2003). Interestingly, IMP can also elicit synergistic responses with other L-amino acids (Nelson et al., 2002). Mutagenesis and receptor modeling studies were able to predict the probable way of IMP mediated synergy (Zhang

et al., 2008), but the receptor system for the IMP is still not completely understood. A major aim of this dissertation was to thoroughly investigate the mechanisms for the detection of various L-amino acids and IMP. The studies described in this dissertation advance our knowledge of the receptor system for the detection of L-amino acids and IMP in the oral cavity.

4.1 Are L-Amino Acids and IMP Detected by Multiple or a Single Receptor?

Summary and Discussion:

In 2002, Nelson et al. first investigated the question of L-amino acid receptors using molecular biology and physiological techniques. While HEK cell expression of the T1r1+T1r3 receptor suggested it is a broadly tuned L-amino acid receptor, it failed to generate responses to many L-amino acids presented alone (Nelson et al., 2002). One such example was the classic umami L-amino acid, glutamate. Additionally, the receptor was also not able to respond to IMP presented by itself. Later, several behavioral studies suggested the involvement of multiple receptors for umami detection, but the receptor system for other L-amino acids was not investigated.

A major focus of this study in Chapter 2 was to investigate the response patterns of L-amino acids and IMP in single TSCs. If a single receptor is involved in the detection of all L-amino acids, and if IMP elicits synergy using the same receptor for all L-amino acids, then a single cell that responds to any L-amino acid should in theory also respond to other L-amino acids and elicit synergistic responses when L-amino acids were presented with IMP. However, we found that response patterns of L-amino acids varied

significantly among TSCs. A majority of single TSCs responded to multiple L-amino acids, but not all L-amino acids elicited responses in the same cell. Moreover, IMP alone elicited responses in a large number of cells, and only a subset of L-amino acid+IMP mix responses was synergistic. These data clearly indicate there are multiple receptor systems for L-amino acid detection. There are probably different receptors that participate in synergistic responses, or just L-amino acid or IMP responses without synergy. Thus, IMP probably acts as an agonist as well as allosteric modulator, depending on receptor type and/or binding site.

A key finding supporting this hypothesis is the response patterns of TSCs in T1r3 KO mice to stimulation by L-amino acids and IMP, reported in the second part of Chapter 2. These cells also generated response patterns somewhat similar to the TSCs of wild type mice, and some cells produced synergy to the mix of L-amino acids and IMP, but the intensity of synergistic responses was much lower compared to wild-type TSCs. Moreover, for wild type mice, there were some cells that responded only to the L-amino acid+IMP mix but not to the individual components. This particular response pattern was completely absent in case of the T1r3 KO mice. While this indicates that T1r1+T1r3 is important for synergistic responses, the presence of synergistic responses in T1r3 KO cells indicated other receptors can also generate synergy. These studies further strengthen the concept that multiple receptors are involved in L-amino acid and IMP detection.

4.2 mGluRs Detect L-Amino Acids and IMP Taste:

Summary and Discussion

In addition to T1r1+T1r3, there are several other candidate receptors for umami taste. Among these, mGluR1 and mGluR4 are outstanding candidates. A truncated N-terminus variant of each of these receptors is found in TSCs. At the same time, brain variants are also present and both variants are probably involved in the umami taste. Chapter 3 focuses on investigations of the functions of these mGluRs in L-amino acids and IMP detection. Activation of TSCs by selective mGluR1 and mGluR4 agonists confirms the existence of these receptors in the TSCs. Moreover the selective antagonists for the receptors suppressed the responses elicited by different L-amino acids and IMP in TSCs of T1r3 KO mice, suggesting these receptors function as L-amino acids and IMP detectors.

Curiously, the effects of mGluR antagonists (*RS*)-1-Aminoindan-1,5-dicarboxylic acid (mGluR1 antagonist; AIDA) and (*RS*)-α-Methylserine-*O*-phosphate (mGluR4 Antagonist; MSOP) were not uniformly antagonistic. Although both AIDA and MSOP significantly suppressed the L-amino acid- and IMP-elicited responses, in some TSCs the agonist (an L-amino acid or IMP) elicited a larger response in the presence of the antagonist, which came back to baseline after wash (Figure 4.1). These types of responses were very random. For example, AIDA exhibited this kind of responses only in case of the T1r3-GFP, but not the T1r3 KO TSCs. On the other hand MSOP exhibited this kind of response in T1r3 KO TSCs. This randomness makes it very difficult to investigate further. Previous studies with mGluRs have reported induced-super sensitivity of these receptors when the antagonists were bath applied before application of the agonist (Yang, Sun, Peng, Zhang, & Yang, 2011). In our case some responses might be caused by

supersensitive receptors. Additionally, it is possible that mGluRs might have a modulatory function in the taste bud that mediate cell-cell signaling. Further investigation is required to test these hypotheses.

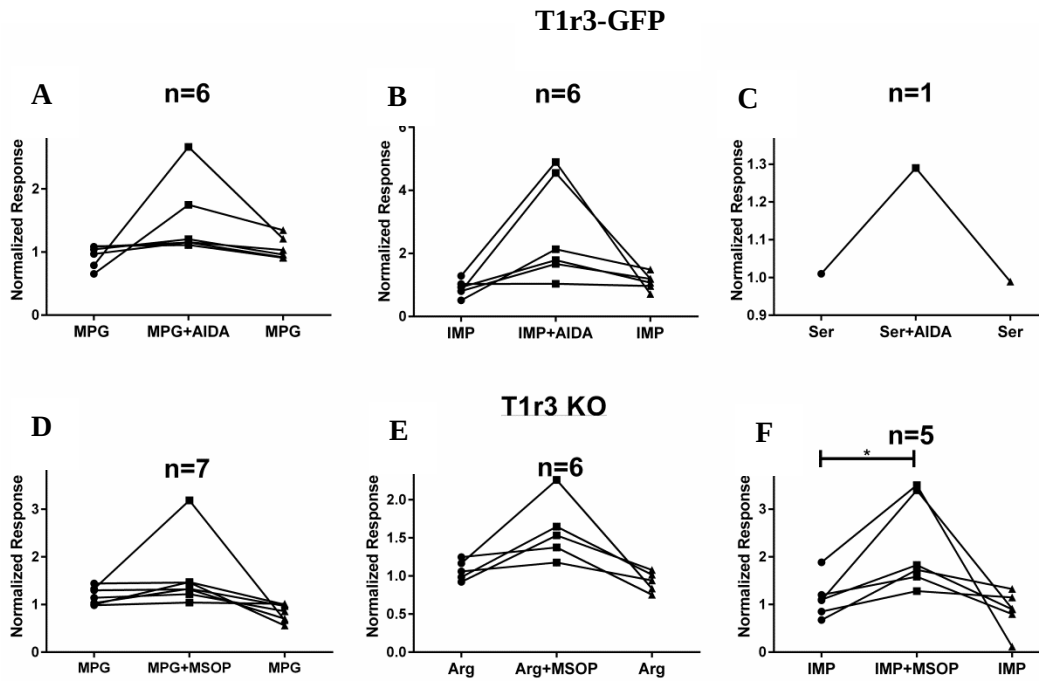

Figure 4.1: Effect of AIDA and MSOP were not uniformly antagonistic.
(A, B, C) Selective mGluR1 antagonist AIDA (100 µM) caused an increase in agonist (MPG (10 mM), IMP (1 mM), and Serine (20 mM)) response, which recovered after wash. (D, E, F) Selective mGluR4 antagonist (100 µM) caused an increase in agonist (MPG (10 mM), IMP (1 mM), and Serine (20 mM)) response, which recovered after wash.

Recently T1rs and T2rs have been found to be expressed in many different tissues, where they mainly function as detectors for nutrients and/or environmental toxins (Li, 2013). These receptors were also implicated in many disease conditions, but our knowledge of the functions of these receptors is very limited. Hence, having a better understanding of the receptor systems will be helpful for understanding the pathophysiology of these disease states.

The concept of the receptor system for detecting L-amino acids commonly held by present day investigators does not explain all the response patterns found in L-amino acid elicited responses. Since these receptors can play important roles in diverse physiological functions in other tissue and are involved in many disease conditions, finding the receptors and their functioning provides opportunities for the development of artificial agonists and antagonists.

4.3 Speculations about possible receptors:

G-protein coupled receptors can often be found to form dimers with each other. It will not be surprising if more heterodimers are found in the near future to act as taste receptors, as redundancy in biological systems is rather common. In the posterior tongue, more T1r1s are expressed compared to T1r3s. However, according to HEK cell expression data, T1r1 individually does not generate any responses to L-amino acids (Nelson et al., 2002). Consequently, it might dimerize with other receptors, such as mGluRs to act as an umami receptor. The binding site for glutamate and IMP, both are on the T1r1 subunit. There might be some yet unidentified receptor(s) which dimerizes with T1r1 at least in the posterior part of the tongue and generates umami responses.

The function of mGluR4 and to some extent mGluR1 is well established in glutamate taste (Lin & Kinnamon, 1999; Eschle, Eddy, & Delay, 2009; Yasumatsu et al., 2009; Nakashima, Eddy, Katsukawa, Delay, & Ninomiya, 2012; Kusuhara et al., 2013; Yasumatsu et al., 2015). However, whether or not they can generate any synergy is not known. Since activation of mGluR4 is generally coupled with a decrease in cAMP concentrations (Flor et al., 1995; Thomsen et al., 1997), and cAMP downregulation is

associated with a possible synergy pathway (Kinnamon, Lin, Ogura, Ruiz, & Delay, 2005; Kinnamon & Vandenbeuch, 2009), mGluR4 alone or in combination with other receptors can function as synergy producing receptor. mGluRs are often found to dimerize with other mGluRs. In the taste system, it is not known if mGluRs dimerize. However, it has been reported recently that mGluR4 can dimerize with mGluR2 in the CNS (Kammermeie, 2012). Tongue epithelium expresses mGluR2/3, but their role in taste transduction, if any, is not known. It is possible that mGluR4 may dimerize with mGluR2 to generate taste responses. Co-immunoprecipitation and in vitro expression studies might be useful to investigate if the above mentioned receptor combinations actually exists and work as functional L-amino acid receptors.

4.4 Preliminary Studies and Future Directions

4.4.1 Preliminary Studies and Future Direction Concerning Chapter 2:

IMP and glutamate bind to the N-terminus domain of T1r1 (Zhang et al., 2008). Thus T1r1 can function as a synergy-producing receptor and possibly as an IMP receptor. However, HEK cells expressing only the T1r1 subunit do not elicit any response to L-amino acids or IMP (Nelson et al., 2002). Hence, it is unlikely to function individually as an L-amino acid and umami receptor. In 2003, Zhao et al. demonstrated complete loss of behavioral and physiological responses to umami in T1r1 KO mice (Zhao et al., 2003). However, they obtained similar results in T1r3 KO mice, which do not agree with T1r3 KO data from other labs, probably because of how the knockouts (KO) were created (Damak et al., 2003). In T1r1 and T1r3 KO created by Zhao et al., only the exons for the N-terminal domain (for T1r3 KO) or the transmembrane domain (for T1r1 KO) were

deleted. Theoretically, this should eliminate the expression of any functional receptor, but if any part of the protein is expressed it might have a dominant negative effect and hamper the function of other receptors. Added to this, these mice were created in a non-taster background, and were backcrossed to C57BL6J background for only 3 generations (as opposed to general 10 generations recommended). This might also explain the complete lack of taste responses in these mice. In contrast, all the exons for *T1r3* gene were deleted in T1r3 KO mice created by Damak et al. (2003). Recently Kusuhara et al. (2013), using their individually generated KO mice in which all exons for *T1r1* gene were deleted, found that their mice were able to generate responses to umami compounds but were not able to produce any synergy. Studying the response pattern of L-amino acids in TSCs of these T1r1 KO mice would be helpful to further strengthen the multiple L-amino acid taste receptor theory.

A preliminary study using TSCs from T1r1 KO mice (Zhao et al., 2003) revealed that these cells can respond to L-amino acids, but the percentage of responsive cells was much lower (only 5%) compared to wild type (28%) or T1r3 KO (15%) mice (Note: The T1r3 mice were the same as those developed and studied by Damak et al., 2003). In these studies, I performed calcium imaging of isolated TSCs. All procedures and solutions were as mentioned in the methods section in Chapter 2 and Chapter 3. Because of the low percentage of responsive cells, a cocktail of L-amino acids (MPG (10 mM), Ser (20 mM), and Gln (10 mM)) was used instead of individual L-amino acids. Additional stimuli included IMP (1 mM), Arg (10 mM), Arg+IMP, and the L-amino acid cocktail+IMP. I found a small number (5%) of the cells responded to at least one stimulus (Table 4.1).

Table 4.1: Summary of responses generated by TSCs of T1r1 KO mice.

Cells Successfully tested with all stimuli	**154**
Cells that responded to at least one stimulus	**8**
Stimuli	**No. of Cells**
IMP	6
L-amino acid cocktail	5
L-amino acid cocktail+IMP	5
Arginine	6
Arginine+IMP	7

This was interesting because, like wild type or T1r3 KO mice, TSCs from T1r1 KO mice also exhibited a diverse response pattern to the bath-applied stimuli (Figure 4.2) and a high percentage of the responsive cells responded to the IMP (Table 4.1). Zhao et al. (2003) reported a complete loss of behavioral and nerve responses in these mice. In addition a recent behavioral study using CTA methods also reported that this strain of knockout mice showed no apparent ability to respond to any L-amino acid, regardless of whether the L-amino acid might elicit a bitter response (E. R. Delay & Kondoh, 2015). The apparent loss of ability to detect L-amino acids by these mice makes the responses that I found unexpected and very intriguing. These Ca^{2+} imaging data may provide another layer of evidence for multiple L-amino acid and IMP receptor theory. It will be interesting to see how the TSCs from T1r1 KO mice from Kusuhara et al. (2013) would respond to different L-amino acids, as that will be helpful to get clear evidence for T1r1 function.

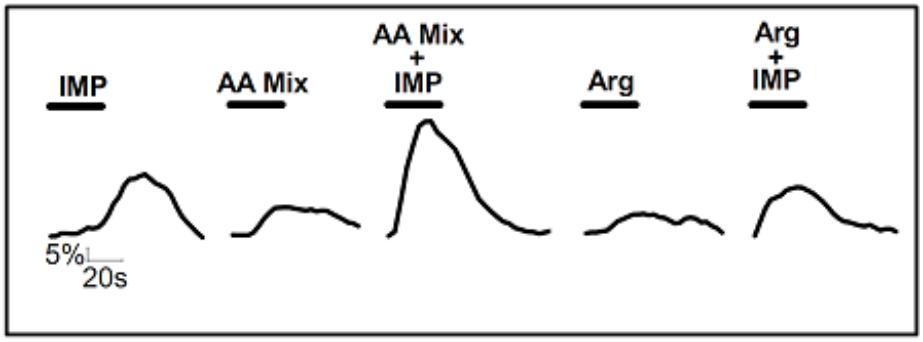

Figure 4.2: Representative Ca^{2+} responses of a TSC from T1r1 KO mice.
Stimuli tested were IMP (1mM), a mix of three L-amino acids (AA: MPG (10 mM) + serine (20 mM) + glutamine (10 mM)), L-arginine (Arg: 10mM), and a mix of amino acid mic with IMP (AA MIX+IMP), and arginine+IMP (Arg+IMP). Amino acids are chosen based on different side-chain groups. The bar above each stimulus trace represents stimulus application time (30 sec).

4.4.2 Can mGluRs Elicit Synergistic Responses?

A synergistic response between L-glutamate and IMP is a characteristic property of umami taste (Sato & Akaike, 1965; Yamamoto et al., 1991; Delay et al., 2000; Wifall, Faes, Taylor-Burds, Mitzelfelt, & Delay, 2007). In Chapter 2 we showed that, like MPG, other L-amino acids elicited synergistic responses in TSCs of T1r3 KO mice when mixed with IMP (Pal Choudhuri et al., 2015). This suggests other receptor(s) can also produce synergy. T1r1+T1r3 is the primary receptor proposed to elicit synergistic umami responses, but it is unclear if mGluRs can also produce synergy. Calcium imaging of isolated TSCs can be performed to see effect of different mGluR selective antagonists on synergistic responses.

The expression of the T1r1+T1r3 heterodimer is not uniform throughout the different taste papillae and is lower in the posterior portion of the tongue (Kim et al., 2003; Kusakabe et al., 2005). In circumvallate papillae, the majority of the T1r3 expressing cells also express T1r1, but only around 50% of the T1r1 expressing cells co-

express T1r3. One possibility is that mGluRs can form dimers with T1r1 and can function as a heterodimer. In future studies, immunohistochemistry can be performed to determine if T1r1 and mGluR4 or mGluR1 are co-expressed in the same call. Co-immunoprecipitation can also be performed to investigate if mGluRs interact with T1r1 and or T1r3.

4.4.3 Are L-Amino Acid and IMP Responses Mediated by PLCβ2 Dependent pathway?

There are questions about the transduction mechanisms for L-amino acids and IMP that need attention. T1r1+T1r3 mediated glutamate transduction is proposed to be signaled by PLCβ2 dependent calcium increase and activation of TrpM5 channel. This is based on the expression of PLCβ2 and TrpM5 in the same cell, reduced responses to glutamate in the PLCβ2 or TrpM5 knockout mice, and inhibition of L-glutamate mediated responses in presence of PLCβ2 inhibitor (Clapp, Stone, Margolskee, & Kinnamon, 2001; Perez et al., 2002; Zhang et al., 2003; Damak et al., 2005; Chandrashekar, Hoon, Ryba, & Zukar, 2006; Maruyama, Pereira, Margolskee, Chaudhari, & Roper, 2006). Interestingly, mice in which the gene sequences of various components of this pathway have been deleted generally do not show a complete loss of responses, suggesting other possible pathways are involved. The possibility that other L-amino acids and IMP transduction are also mediated by the same pathway has not been studied. Pharmacological inhibitors of PLCβ2 pathway could be used to see if L-amino acids and IMP responses are blocked these inhibitors.

4.4.4 Is cAMP Pathway Involved in L-Amino Acid and IMP Detection?

$G\alpha_i$ has been found in TSCs. $G\alpha_i$ generally causes downregulation of cAMP by activation of phosphodiesterase (Rarick, Artemyev, & Hamm, 1992; Clapp et al., 2008). Glutamate stimulation of TSCs has been shown to cause a decrease in the cAMP level in forskolin (adenylase cyclase activator) treated taste epithelium (Abafy, Trubey, & Chaudhari, 2003). To determine if IMP and other L-amino acids with or without IMP, can affect the cAMP levels in TSCs, I performed immune assays for cAMP detection using a cAMP direct Biotreck ELISA kit. Some TSCs are proposed to be functionally heterogeneous and may contain signaling components for responding to multiple different stimuli. Thus, disruption of taste tissue for an immunoassay procedure can cause interactions between non-physiological combinations of receptors with G-proteins (as G-proteins can be found in membrane as well as in intracellular compartments). This may result in the activation of effector enzymes that will not occur in the intact taste tissue. To avoid this, stimuli were applied to intact taste cells before cAMP measurements.

Taste epithelium was collected as controlled pair (Figure 4.3A) and incubated with forskolin for 10 minutes prior to stimulus application to elevate cAMP level. cAMP was measured with and without stimulation with effective concentrations of stimuli. No change was observed in the non-taste epithelium (Figure 4.3). IMP stimulation had no effect on the cAMP levels (Figure 4.3B). Interestingly, serine and arginine (with or without IMP) showed variable results (Figure 4.3C, D, E, F). For example, serine and serine+IMP, both exhibited a decrease in the cAMP levels for circumvallate papillae, but serine+IMP showed an increase in the cAMP levels in TSCs of foliate papillae. Arginine

and arginine+IMP, both caused a decrease in cAMP level in foliate papillae, but arginine caused a slight increase in cAMP levels in circumvallate taste buds (Figure 4.3). These differences in the responses might explain how a single L-amino acid elicits multiple taste qualities (Kawai et al., 2012) but further experimentation is needed to investigate this hypothesis.

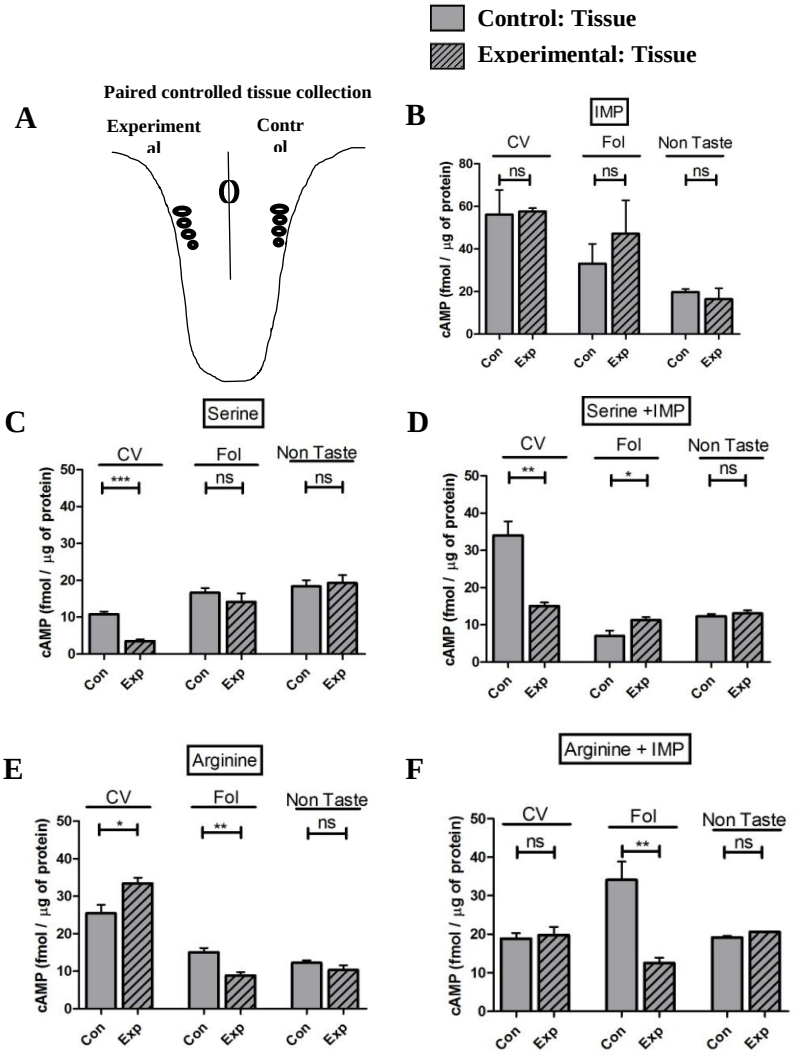

Figure 4.3 Taste stimuli can cause a change in cAMP level in TSCs.
(A) Cartoon of a tongue showing how paired control tissue was selected. Control and experimental both tissue were collected from same mice. Only taste epithelium was collected. (B, C, D, E, F) Tissues were

incubated with FSK for 10 min prior to stimulus application to induce cAMP production. Activation of taste papillae by IMP (1 mM), or L-amino acids (serine (20 mM), arginine (10 mM)) caused a change in cAMP level in some experiments. No change was observed for non-taste epithelium. CV, circumvalette; Fol, Foliate; FSK, Forskolin.

4.4.5 Does cAMP Pathway Interact with PLCβ2-Mediated Pathway?

Although the intracellular signaling pathway for synergistic responding is not yet understood, one hypothesis involves the downregulation of cAMP pathway, which in turn causes the downregulation of PKA and subsequent release of phosphorylation and further activation of the PLC-mediated pathway (Kinnamon, Lin, Ogura, Ruiz, & Delay, 2005; Kinnamon & Vandenbeuch, 2009). This is partially based on the fact that Gα gustducin knockout mice exhibit highly elevated basal levels of cAMP in taste cells (Clapp et al., 2008). To test this hypothesis, I performed Ca^{2+} imaging of isolated TSCs to explore if H-89, a cAMP dependent protein kinase inhibitor, would have any effect on L-amino acid- and IMP-mediated responses. H-89 was applied for 2 minutes prior to and with the test stimulus. Interestingly, H-89 alone triggered an increase in Ca^{2+} level in L-amino acid or IMP responsive TSCs (Figure 4.4). In MPG, serine, and serine+IMP responsive cells, H89 elicited large responses. In serine+IMP responsive cells, the H89 response was significantly greater than the serine+IMP response. But in every case, the addition of the stimulus with H89 was unable to cause any further increase in Ca^{2+} level. Application of H89 for 2 minutes might have caused a ceiling effect for Ca^{2+} increases. Thus, an additional increase in Ca^{2+} level might not be possible. Nevertheless, these experiments suggest the interactions between cAMP and PLC mediated pathways in L-amino acid responsive TSCs. Additional studies are needed to explore the role of the interaction of these pathways in synergistic responses.

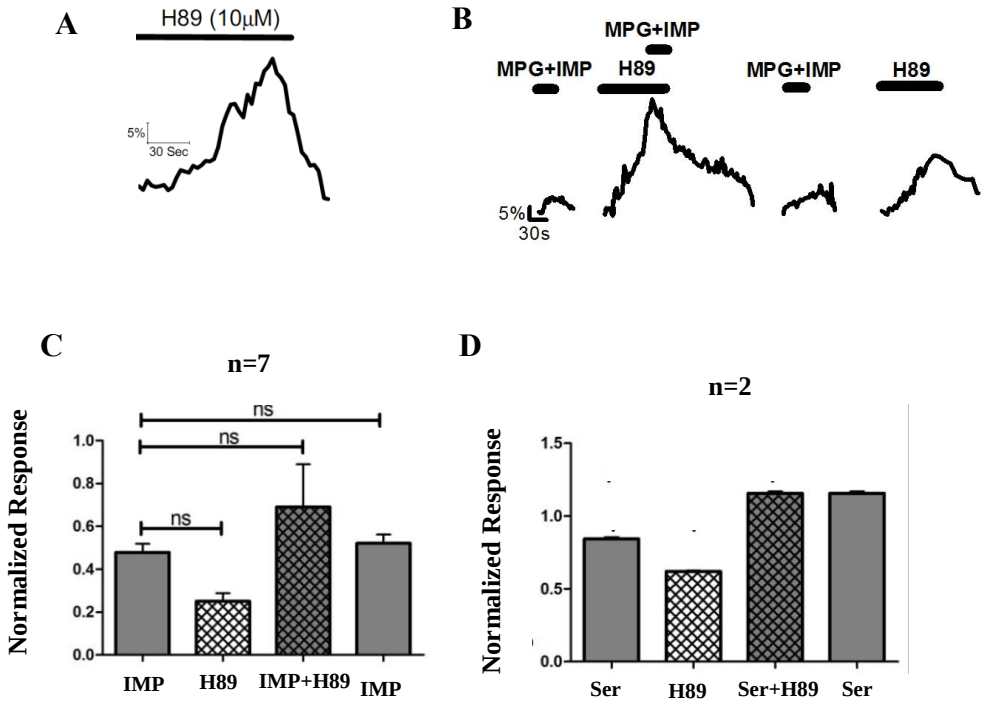

Figure 4.4: Effect of H89 on L-amino acids and IMP elicited responses.
H-89 was applied 2 min prior to and with stimulus. (A, B) H-89 (10 μM) alone elicited large responses in TSC. (B, C, D) H89 was not able did not had any effect on the IMP (1 mM), or Serine (Ser- 20 mM) elicited responses probably due to the ceiling effect. Repeated measure ANOVA followed by Tukey's test was used for statistical testing

4.5 Conclusions

In summary, our data indicate multiple receptor function in the detection of L-amino acid and IMP taste and in generating synergy. Along with T1r1+T1r3, mGluRs (1 and 4) can also mediate L-amino acid and IMP responses. Supplementation of L-amino acids to various food substances, particularly in sport drinks and protein drinks, is increasing in popularity. However, one major challenge for food industry is to keep these drinks palatable without added sugar. In this case the synergistic taste enhancement properties of L-amino acids in presence of IMP can be put into good use with a careful

combination of various L-amino acids and IMP. Additionally the combination of L-amino acids can also be used to increase the palatability of low salt, low fat, and/or low sugar diet.

A new direction of taste research involves the understanding the function of taste receptors in the various other tissues, especially in the gut. This is probably important because it acts as a way of detecting chemical signals from the lumen, and regulates hormonal activity. Further study of these pathways will help us understand the function of the gut in appetite control and digestive processes.

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